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p57^{Kip2} regulates the proper development of labyrinthine and spongiotrophoblasts

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The cyclin-dependent kinase (cdk) inhibitor, *p57^{Kip2}* is a tumour suppressor candidate and a paternally-imprinted gene. In humans, the *p57^{Kip2}* gene is located on chromosome 11p15.5, a region implicated in both sporadic cancers and Beckwith–Wiedemann syndrome. From analysis of p57^{Kip2}-deficient mice, we demonstrate the relationship between trophoblastic abnormalities and p57^{Kip2}. Both *p57^{Kip2}* null ($^{-/-}$) embryos and heterozygous embryos with a maternally-derived mutated allele ($^{+*/-}$) displayed placentomegaly, as well as dysplasia of labyrinthine and spongiotrophoblasts. The number of labyrinthine trophoblasts of homozygous embryos was twice that in wild-type embryos. When we measured kinase activities of cdk in total placenta lysates by the immuno complex kinase assay, there were no differences among the genotypes. These results show that p57^{Kip2} may function in the proper development of labyrinthine and spongiotrophoblasts by pathways that are not involved with regulation of cdk activities. It is, therefore, suggested that p57^{Kip2} protein might have an unknown role.

Key words: Beckwith–Wiedemann syndrome/choriocarcinoma/cyclin-dependent kinase inhibitor/imprinting/tumour suppressor gene

Introduction

The cyclin-dependent kinase (cdk) inhibitor, p57^{Kip2} is a tumour suppressor. It has the ability to bind with a variety of cyclin–cdk complexes and to inhibit their kinase activities *in vitro*. p57^{Kip2} belongs to the Cip/Kip family, and shares homology with p21^{Cip1} and p27^{Kip1} at the *N*-terminal domain (cdk-binding/inhibitory domain). It is distinguished from p21^{Cip1} and p27^{Kip1}, however, by its unique domains: a proline-rich domain and an acidic domain in mouse p57^{Kip2}, and a PAPA domain in human p57^{Kip2}. As is the case with p21^{Cip1} and p27^{Kip1}, the over-expression of p57^{Kip2} causes cells to arrest in the G_1 phase. In contrast to the widespread expression of p21^{Cip1} and p27^{Kip1}, p57^{Kip2} is expressed in a tissue-specific manner (Lee *et al*., 1995; Matsuoka *et al*., 1995). The location of the *p57^{Kip2}* gene both in humans (chromosome 11p15.5) and mice (distal chromosome 7) is in a cluster of imprinted genes including *IGF-II* and *H19*. Both *H19* and *p57^{Kip2}* are expressed from the maternally-derived allele, whereas *IGF-II* is expressed from the paternally-derived allele (Hatada and Mukai, 1995; Matsuoka *et al*., 1996; Taniguchi *et al*., 1997).

In humans, the loss of the maternally-derived 11p15.5 region is implicated in both sporadic tumours and Beckwith–Wiedemann syndrome (BWS), which is characterized by congenital malformations and organomegaly associated with an increased risk for the development of childhood neoplasms (Reik and Maher, 1997). Abnormal expression of imprinted genes induces the pathogenesis of certain paediatric tumours, including Wilms' tumour, rhabdomyosarcoma, and trophoblastic tumour (Hoovers *et al*., 1995).

Mutations of the *p57^{Kip2}* gene have been found among BWS patients, although only rarely. In Wilms' tumour tissues, no mutations of the *p57^{Kip2}* gene have been detected (Hatada *et al*., 1996; O'Keefe *et al*., 1997; Bhuiyan *et al*., 1999). Nevertheless, a decrease of p57^{Kip2} expression levels has been detected in Wilms' tumour tissues, adrenal tumour tissues, and cultured adrenocortical cells (Chung *et al*., 1996; Thompson *et al*., 1996; Liu *et al*., 1997). Therefore, p57^{Kip2} might function as a tumour suppressor.

Choriocarcinoma is the most malignant form of trophoblastic tumour. The risk of choriocarcinoma is 2000–4000-times greater after a molar conception than after normal pregnancy. The mole, which is characterized by the absence of the maternal genome and grossly swollen villi in the absence of a fetus, is an example of the vital importance of genomic imprinting. From recent studies, it has been revealed that a high level of H19 and/or over-expression of IGF-II resulting from the loss of imprinting, induces the development of choriocarcinoma (Ariel *et al*., 1994; Walsh *et al*., 1995; Arima *et al*., 1997; He *et al*., 1998). Since imprinted genes, including *IGF-II*, *H19* and *p57^{Kip2}*, are associated with trophoblastic disease, it is possible that *p57^{Kip2}* may regulate the disease.

Mice deficient in the *p57^{Kip2}* gene have shown defective endochondral bone formation. Most of those that died neonatally displayed cleft palates (Yan *et al*., 1997; Zhang *et al*.,

1997; Takahashi *et al*., 2000). Zhang *et al*. (1997) reported the birth of $p57^{Kip2}$-deficient neonates that displayed organomegaly and abdominal wall defects (two of the main hallmarks of BWS), and no mice survived beyond the neonatal period. On the other hand, Yan *et al*. (1997) and Takahashi *et al*. (2000) reported the $p57^{Kip2}$-deficient mice that displayed none of the hallmarks of BWS, and 10% of them survived. Yan *et al*. reported that these mice did not display any abnormalities, whereas the mice reported by Takahashi *et al*. displayed growth retardation. These reports of surviving mutant mice did not indicate whether the $p57^{Kip2}$-deficient mice had tumour tissues.

Here we demonstrate trophoblastic dysplasia in the chorio-allantoic placentae of mouse embryos lacking $p57^{Kip2}$. Zhang *et al*. (1998) also reported that the mice with double mutations of both $p57^{Kip2}$ and $p27^{Kip1}$ as well as $p57^{Kip2}$-deficient mice displayed abnormal placental development. We also showed that $p57^{Kip2}$, together with the imprinted genes, may function in the proper development of labyrinthine and spongiotrophoblasts. In this report, it is suggested that $p57^{Kip2}$ may regulate the activities of molecules other than cdk, at least in the placenta, as there are no differences in cdk activities of the placenta lysates from the genotypes examined.

Materials and methods

Mice

The mice used in this study, which carry a targeted mutation in the $p57^{Kip2}$ loci, were created in the Nippon Roche Research Center (Takahashi *et al*., 2000). The materials derived from the $p57^{Kip2}$-deficient mice were provided by Nippon Roche K.K. Kanagawa, Japan and were used for exploratory research only. The genotypes of the mice were determined by polymerase chain reaction (PCR). The $p57^{Kip2}$ locus is imprinted, and is expressed from the maternally-derived allele. The heterozygotes expressing $p57^{Kip2}$ were derived from the mating between heterozygous males and wild-type females. The heterozygous embryos lacking $p57^{Kip2}$ expression were derived from the mating between wild-type males and heterozygous females. To avoid confusion, we indicated the imprinted allele from paternal origin by adding an asterisk after the + symbol, for example, $p57^{+*/-}$ indicates the heterozygote without $p57^{Kip2}$ expression.

Gross and histological analysis

Placentae (17.5 days post-coitum) were fixed in 4% paraformaldehyde. For histological analysis, fixed samples were dehydrated through ascending concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Embedded samples were sectioned at 5 μm, and each section was retrieved from the water bath. Sections were stained with haematoxylin and eosin, dehydrated and cleared. They were viewed with an Olympus AH3 microscope (Olympus, Tokyo).

Counting of placental cells

Counting of placental cells was performed as described (Lopez *et al*., 1996). Briefly, cells were counted in representative sections using an ocular grid. Micrographs of three sections of each tissue were used to count and compare cell numbers in morphologically equivalent areas of each placenta. A middle section that contained both basal and labyrinth parts was selected as the first section.

In-situ hybridization

Extracted placentae were immediately frozen in a bed of pulverized ice. Cryosections were cut at 12–20 μm and thaw-mounted on poly-L-lysine-coated slides, fixed in phophate-buffered saline (PBS) containing 4% paraformaldehyde, and acetylated with acetic anhydride. A digoxigenin-labelled riboprobe was generated from mouse $p57^{Kip2}$ cDNA, inserted into pBluescript and kindly supplied by S.Matsuoka (Matsuoka *et al*., 1995). Hybridization was performed at 50°C overnight with 0.25 mg/ml of the riboprobe diluted in hybridization buffer, containing 5× sodium chloride/sodium citrate (SSC), 50% formamide, 5× Denhardt's solution, 250 mg/ml total yeast RNA, and 500 mg/ml DNA of herring sperm. Sections were then subjected to low-stringency (2× SSC) and high-stringency (0.1× SSC/50% formamide at 55°C) washing. Hybridized riboprobe was detected with an alkaline phosphatase-coupled anti-digoxygenin antibody (Boehringer Mannheim). Naphthol-AS-MX phosphate was used as chromogen.

Immunoblot analysis

Total lysates (200 μg) of placentae were prepared with Tween 20 lysis buffer (Nakayama *et al*., 1996). The protein concentration of the total lysates was determined by the Bradford method (protein assay; Bio-Rad, Kentucky, USA). Total lysates were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 9% gel for the detection of $p27^{Kip1}$, $p57^{Kip2}$, and proliferating cell nuclear antigen (PCNA) or on a 12% gel for the detection of cyclin-dependent kinase (CDK)2 and CDK4. They were transferred to Immobilon-P membranes (Millipore, California, USA). The membranes were probed with either polyclonal antisera against mouse $p57^{Kip2}$ (Takahashi *et al*., 2000) or monoclonal antibodies against p27Kip1 (Transduction Laboratories, Massachusetts, USA), PCNA (Santa Cruz, California, USA), CDK2 (Santa Cruz), and CDK4 (Santa Cruz). Proteins were visualized by ECL (Amersham).

Immuno complex kinase assay for cdk2 and cdk4

Total lysates (200 μg) of placentae prepared with Tween 20 lysis buffer were incubated with either rabbit anti-CDK2 antibody (Santa Cruz) or rabbit anti-CDK4 antibody (Santa Cruz) for 3 h on ice. Immunocomplexes bound to protein A–Sepharose were washed with Tween 20 lysis buffer. For the kinase assay, the Sepharose beads with complexes were washed with 50 mmol/l HEPES (pH 7.5) and suspended in 30 μl of kinase buffer [50 mmol/l HEPES pH 7.5, 10 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, 10 μCi of (γ-^{32}P)-ATP (6000 Ci/mmol; Amersham) and 0.1 mmol/l glutathione] with freshly prepared *Escherichia coli*-expressed glutathione *S*-transferase(GST)-Rb proteins (Matsushime *et al*., 1994). The samples were incubated for 20 min at 30°C, denatured in SDS sample buffer, and applied to a 10% SDS–PAGE. Dried gels were exposed with STORM (Molecular Dynamics, California, USA).

Results

Placentomegaly in $p57^{Kip2}$-deficient embryos

To investigate whether $p57^{Kip2}$-deficient mouse embryos have placentomegaly, we measured the wet weights of placentae of embryos derived from heterozygote matings. As shown in Figure 1a, placentae of $p57^{Kip2}$ homozygotes ($p57^{-/-}$) grew larger than those of wild-type mice. We also examined the somatic growth of embryos, but detected no difference in the body weights of embryos between genotypes. This placentomegaly of $p57^{-/-}$ was also observed in $p57^{+*/-}$ embryos (Figure 1b). In mice, the chorio-allantoic placenta comes into operation around mid-gestation. We used the term 'placenta' as shorthand for the chorio-allantoic placenta below.

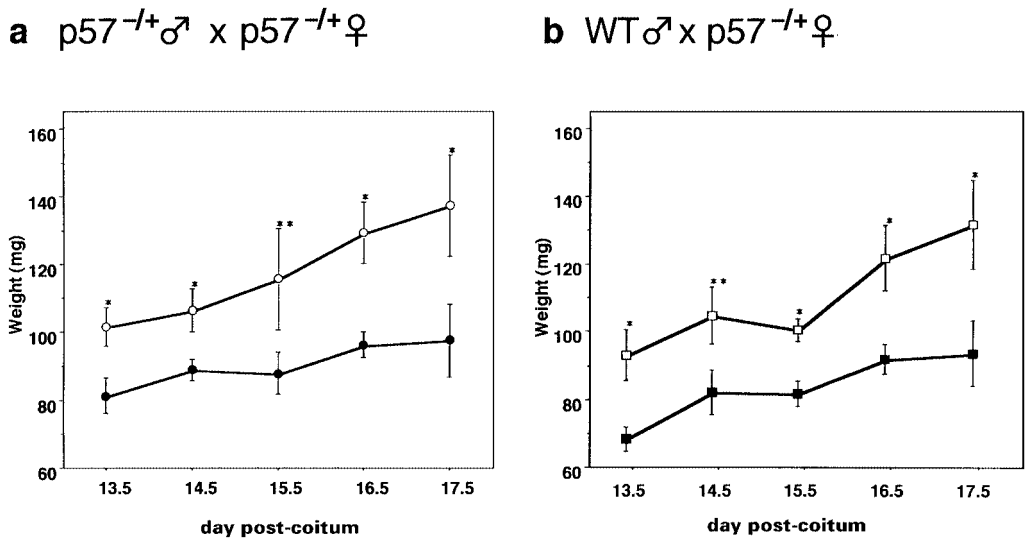

Figure 1. Placentomegaly in p57^{Kip2}-deficient mouse embryos. The p57^{Kip2}-deficient mouse embryos, p57$^{-/-}$ and p57$^{+*/-}$, displayed placentomegaly. Placentas were resected from the pregnant p57$^{-/+}$ female mice at the days post-coitum indicated. (**a**) Weights of p57$^{-/-}$ placentae (○) were compared with those of wild-types (WT; ●). These were derived from the p57$^{-/+}$ matings. (**b**) The weight of p57$^{+*/-}$ placentae were compared with those of wild-type ones. These were yielded from the crosses between wild-type male and p57$^{-/+}$ female. The paternally-derived p57^{Kip2} gene was imprinted, and the heterozygotes (p57$^{+*/-}$; □) derived from these crosses could not express p57^{Kip2} protein. (■ = wild-type). In both (**a**) and (**b**), at each day post-coitum, the weights of the p57$^{-/-}$ placentae were significantly different from those of wild-type placentae (controls; $*P < 0.01$, $**P < 0.05$).

Trophoblastic dysplasia in p57^{Kip2}-deficient mouse embryos

The histopathological analysis of these enlarged placentae indicated that p57$^{-/-}$ embryos showed prominent proliferation of labyrinthine and spongiotrophoblasts resulting in thickened placentae and narrowed interlabyrinth spaces (Figure 2). Fibrin deposition of the intervillous space was also observed. These dysplasias in trophoblastic cells were also observed in half of the heterozygous embryos derived from heterozygote matings. Since the *p57^{Kip2}* gene is one of the imprinted genes and both *p57$^{-/-}$* and *p57$^{+/-}$* embryos displayed placentomegaly (Figure 1), we investigated the *p57$^{+*/-}$* and *p57$^{-/+}$* embryos. Whereas *p57$^{+*/-}$* showed moderately enhanced proliferation of trophoblasts (Figure 2c–f), p57$^{-/+}$ were similar to the wild-type embryos (Figure 2a,b), and showed no enhanced proliferation of the trophoblastic cells.

The numbers of labyrinthine and spongiotrophoblasts were significantly increased in p57$^{-/-}$ and p57$^{+*/-}$ embryos compared with wild-type (Tables I and II). The numbers of labyrinthine and spongiotrophoblastic cells in p57$^{-/-}$ (Table I) and p57$^{+*/-}$ (Table II) embryos were twice as many as those of wild-type. The growth of glycogen cells is controlled by IGF-II (Lopez *et al*., 1996), and there were no significant differences in the numbers of either glycogen cells or giant cells among the genotypes. These cells, in which the *p57^{Kip2}* expression was not detectable, were not affected by *p57^{Kip2}* disruption (Table I).

p57^{Kip2} expression in trophoblastic cells

To estimate the *p57^{Kip2}* expression in the trophoblastic cells of our *p57^{Kip2}*-deficient mice, we carried out in-situ hybridization. Digoxigenein-labelled riboprobe was generated from mouse *p57^{Kip2}* cDNA inserted into pBluescript. In the hybridization with the antisense probe, trophoblasts of wild-type embryos showed positive signals, and with the sense riboprobe, the results were negative. In both labyrinthine and spongiotrophoblasts of wild-type embryos, *p57^{Kip2}* expression was recognized (Figure 3a and c). The labyrinthine trophoblasts are strands of cells separated by gaps containing maternal blood, and the spongiotrophoblasts are relatively large cells having circular nuclei. The *p57^{Kip2}* mRNA expression in trophoblastic cells of each genotype was also investigated. In the p57$^{-/+}$, the expression of *p57^{Kip2}* was detected, similar to that in the wild type (Figure 3d); however, it was not detected in either p57$^{-/-}$ embryos (Figure 3e) or p57$^{+*/-}$ embryos (Figure 3f). Thus, *p57^{Kip2}* expression coincided with trophoblastic dysplasia for each genotype. Hatada and Mukai (1995) reported that the *p57^{Kip2}* gene is expressed from the maternally-derived allele in mice. We can confirm that the *p57^{Kip2}* gene was imprinted and expressed from the maternally-derived alleles in trophoblasts from *p57^{Kip2}*-deficient mice.

No effect of p57^{Kip2} deficiency on cdk activities in placental lysates

Since p57^{Kip2} is a cdk inhibitor, we supposed that an increase in cdk activity caused by the lack of p57^{Kip2} would result in the trophoblastic dysplasias as found previously (Zhang *et al*. 1998; Figure 2). To confirm these possibilities, we measured the expression and kinase activities of CDK2 and CDK4, both of which are active in the G_1–S phase transition. In an immunocomplex kinase assay, we used the *E.coli*-expressed GST fusion protein with the *C*-terminal fragment of the Rb protein as the substrate of cdks. We found that the expression levels of both CDK2 and CDK4 were similar in the wild-type and $^{-/-}$, and there was no difference in their activities, regardless of their genotypes (Figure 4b,c). Therefore, the kinase activities of CDK2 and CDK4 were not affected by the disruption of

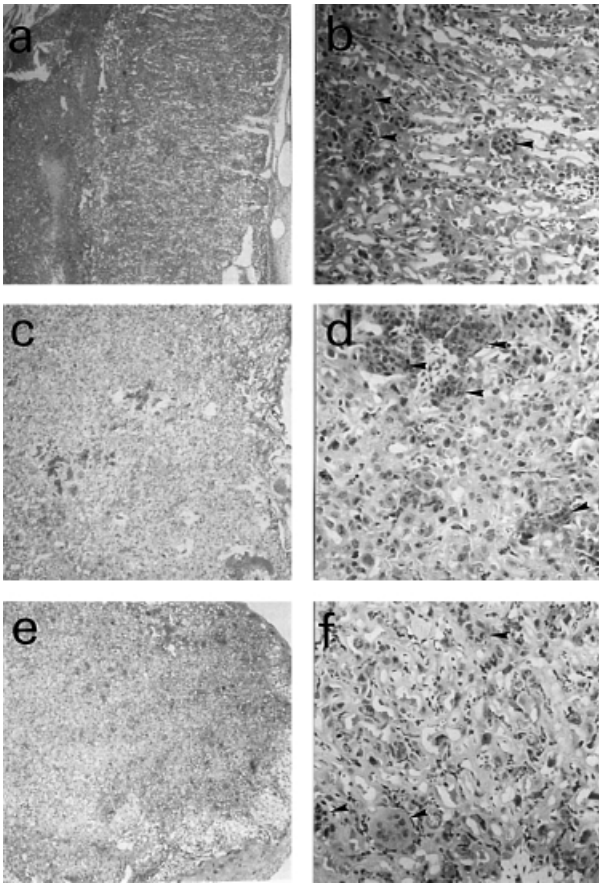

Figure 2. Trophoblastic dysplasia in p57^{Kip2}-deficient embryos. The labyrinthine and spongiotrophoblasts of both p57$^{-/-}$ and p57$^{+*/-}$ embryos were more significantly increased than those of wild-type embryos. (**b**, **d**, and **f**) arrows show spongiotrophoblasts. (**a**, **b**) placenta (17.5 days post-coitum) from wild-type; (**c**, **d**) placenta (17.5 days post-coitum) from p57$^{+*/-}$; (**e**, **f**) placenta (17.5 days post-coitum) from p57$^{-/-}$; (**a**, **c**, **e**) original magnification ×40; (**b**, **d**, **f**) original magnification ×200.

Table I. Mean number of placental cells in p57$^{-/+}$ male×p57$^{-/+}$ female. Three sections from each of three different placentae were used for the analysis of each genotype

	p57$^{-/-}$	wild-type
Labyrinthcytotrophoblast	674 ± 67	334 ± 29
(*P* value)	(<0.005)	
Spongiotrophoblast	2420 ± 185	1296 ± 118
(*P* value)	(<0.005)	
Glycogen cell	1081 ± 80	992 ± 109
	NS	
Giant cell	18 ± 5	15 ± 4
	NS	

NS = not significantly different.

p57^{Kip2} protein. In addition, Figure 4a also demonstrated that disruption of p57^{Kip2} protein expression did not affect the expression levels of p27^{Kip1} protein.

Discussion

We confirmed the occurrence of placentomegaly with trophoblast dysplasia in *p57^{Kip2}*-deficient mice. These phenotypes

Table II. Mean number of placental cells in wild-type male×p57$^{-/+}$ female. Three sections from each of three different placentae were used for the analysis of each genotype

	p57$^{+*/-}$	wild-type
Labyrinthcytotrophoblast	706 ± 77	352 ± 52
(*P* value)	(<0.005)	
Spongiotrophoblast	2063 ± 194	1202 ± 72
(*P* value)	(<0.005)	
Glycogen cell	894 ± 109	863 ± 73
	NS	
Giant cell	16 ± 2	15 ± 1
	NS	

NS = not significantly different.

coincided with the *p57^{Kip2}* expression pattern. These results showed that the p57^{Kip2} protein is an important regulator of labyrinthine and spongiotrophoblast proliferation in mice.

The *p57^{Kip2}* gene is one of the imprinted genes, and is located within the cluster of imprinted genes both in humans (chromosome 11p15.5) and in mice (distal chromosome 7) (Hatada and Mukai, 1995; Matsuoka *et al*., 1996; Taniguchi *et al*., 1997). This cluster also includes *IGF-II* and *H19*. These imprinted genes might be associated with the development of placentae and trophoblastic malignancy. The linkage of BWS with the imprinted genes in the 11p15.5 region has been demonstrated, and *IGF-II*, *H19* and *p57^{Kip2}* have been identified as responsible candidates (Hoovers *et al*., 1995). Placentomegaly is counted as one of the hallmark symptoms of BWS patients (McCowan and Becroft, 1994). Here, the evidence in *p57^{Kip2}*-deficient embryos with placentomegaly has suggested that the *p57^{Kip2}* gene might be recognized as being one of the responsible genes for BWS.

The phenotypes of the *p57^{Kip2}*-deficient mice have been reported by three groups. Zhang *et al*. (1997) reported that the mutant mice displayed organomegaly and abdominal wall defects, both of which are features of BWS, but Yan *et al*. (1997) and Takahashi *et al*. (2000) reported that such mice showed phenotypes that were not features of BWS, i.e. the p57^{Kip2}-deficient mice reported both by Yan *et al*. and by Takahashi *et al*. did not display somatic overgrowth, macroglassia, and abdominal wall defects. From these studies of the *p57^{Kip2}*-deficient mice, it has been debatable whether *p57^{Kip2}* is a gene responsible for BWS. Nevertheless, as we demonstrated in this study, *p57^{Kip2}*-deficient mice showed placentomegaly. Although the imprinting mechanism are largely unclear, it is likely that the expression of genes including *H19* and *IGF-II* within this region could be affected by the deletion of the *p57^{Kip2}*-coding region (or possibly by the insertion of the neomycin-resistant gene cassette).

Similar to *p57^{Kip2}*, *H19* is also known to be expressed from the maternally derived allele. Some groups have reported that H19 expression is enhanced in choriocarcinoma, placental site trophoblastic tumours, and cultured choriocarcinoma cell lines (Ariel *et al*., 1994; Walsh *et al*., 1995; Arima *et al*., 1997; Lustig-Yariv *et al*., 1997). Biallelic expression of H19 was detected in these tumours. Furthermore, the H19 expression level in tumour tissues formed in mouse tumour models by the injection of choriocarcinoma cell lines was unrelated to

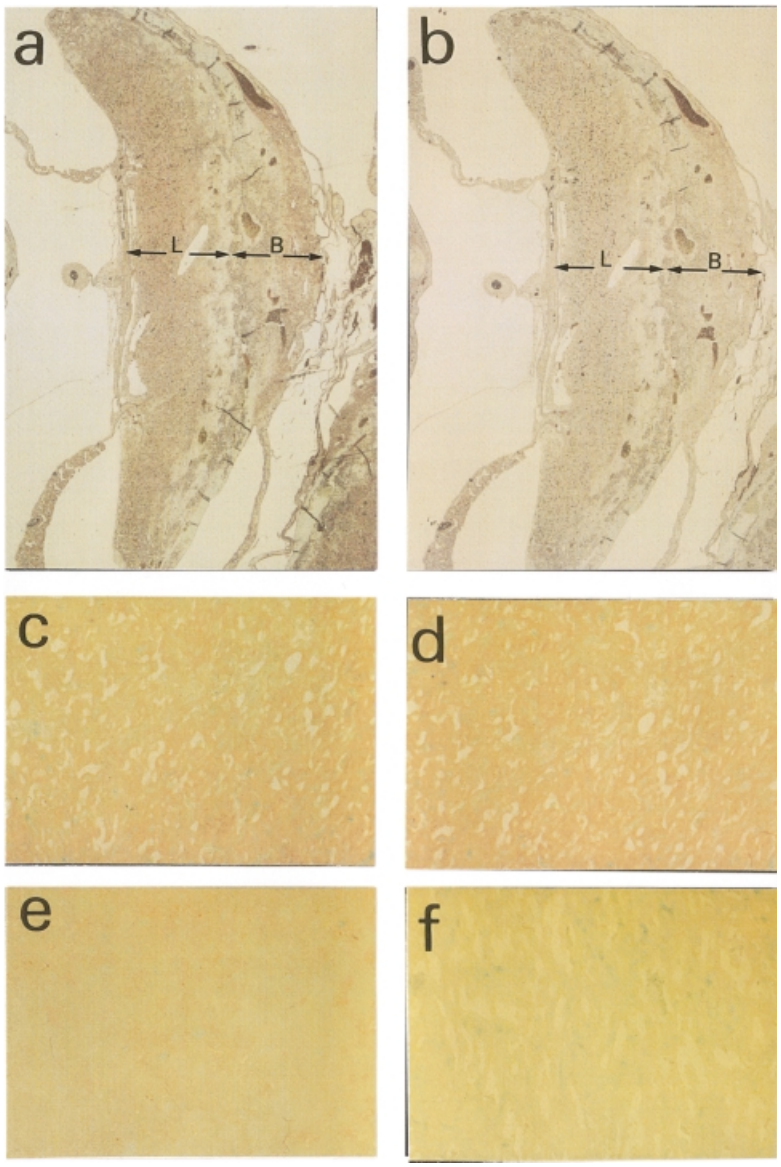

Figure 3. p57^{Kip2} expression in mouse trophoblasts by in-situ hybridization. In the labyrinthine and spongiotrophoblasts of wild-type mouse embryos, p57^{Kip2} expression is indicated by intense staining. In those of p57$^{-/-}$ and p57$^{+*/-}$ embryos, p57^{Kip2} expression was not detectable. (**a** and **b**) L = labyrinthine layer; B = basal layer. (**a–c**) placenta (17.5 days post-coitum) from wild-type; (**d**) placenta (17.5 days post-coitum) from p57$^{-/+}$; (**e**) placenta (17.5 days post-coitum) from p57$^{+*/-}$; (**f**) placenta (17.5 days post-coitum) from p57$^{-/-}$; (**a** and **c–f**) results of hybridization with anti-sense mouse p57^{Kip2} riboprobe; (**b**) result of hybridization with sense mouse p57^{Kip2} riboprobe; (**a**, **b**) original magnification ×20; (**c–f**) original magnification in labyrinthine layers ×200.

that in the cell before the injection. It was suggested that the over-expression of H19 in trophoblasts is involved in choriocarcinogenesis, and therefore, other products of imprinted genes might act as inhibitors of choriocarcinogenesis. *H19* mutant mice also displayed overgrowth derived from the excess of IGF-II but did not stimulate other aspects of the BWS phenotypes, characterized by macrosomia, abdominal wall defects, macroglassia, and other manifestations (Elliot *et al*., 1994; Leighton *et al*., 1995). In mice carrying a maternally-derived 13 kb deletion mutation, which eliminates *H19* and 10 kb of upstream sequence, the normally silent maternal IGF-II allele becomes transcriptionally active by imprint relaxation (Leighton *et al*., 1995). Mice that over-expressed *IGF-II* were reported to exhibit phenotypes of BWS (Eggenschwiler *et al*., 1997; Sun *et al*., 1997). Eggenschwiler *et al*. reported that the mice with mutations in the *IGF-II receptor* gene and *H19* gene have high concentrations of IGF-II as a result of biallelic *IGF-II* gene expression (due to imprint relaxation) and this caused somatic overgrowth, visceromegaly, placentomegaly, omphalocele, and cardiac and adrenal defects. In the *p57^{Kip2}*-mutant mice, neither over-expression of IGF-II protein nor depression of H19 expression were detected (data not shown). Among the genotypes of embryos, we could not recognize differences in the proliferation of glycogen cells that are affected by the IGF-II concentration (Table I). Therefore, we may conclude that placentomegaly in the p57^{Kip2}-deficient mice was caused by mechanism(s) other than IGF-II over-expression.

p57^{Kip2} is one of the cdk inhibitors belonging to the Cip/Kip family, which includes p21^{Cip1} and p27^{Kip1}. We assumed

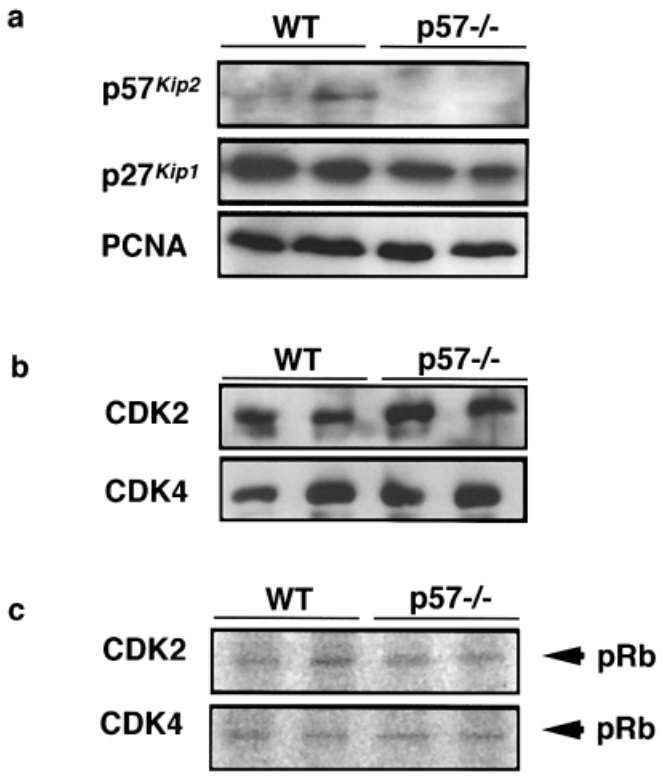

Figure 4. Expression and kinase activities of cyclin-dependent kinase (CDK)2 and CDK4 were not affected by a lack of $p57^{Kip2}$. (**a**) Immunoblotting of $p57^{Kip2}$, $p27^{Kip1}$, and proliferating cell nuclear antigen (PCNA) in placentae from wild-type (WT) and $p57^{-/-}$ mice. Among the Cip/Kip family, $p21^{Cip1}$ was not detectable (data not shown). (**b**) Immunoblotting of CDK2 and CDK4 in mouse placentae. (**c**) Kinase activities of CDK2 and CDK4 in mouse placentae. pRb = phosphorylated GST-Rb protein.

that the placentomegaly in $p57^{Kip2}$ deficient mice resulted from the activation of cdk. Therefore, we measured the kinase activities of both CDK2 and CDK4, which contribute to the G_1–S transition of the cell cycle. In placentae, the kinase activities of both CDK2 and CDK4 were not affected by the disruption of the $p57^{Kip2}$ protein. In mouse embryonic fibroblasts, the disruption of $p57^{Kip2}$ protein does not affect the activities of cdk (Takahashi *et al*., 2000). These results suggest that $p57^{Kip2}$ protein may have a biological activity other than inhibition of cdk activities. It is known that human $p57^{Kip2}$ protein binds PCNA, a DNA replication factor (Watanabe *et al*., 1998). In human trophoblastic diseases, PCNA expression was reported to be increased, although the detection of PCNA might not be useful for clinical prediction (Molykutty *et al*., 1998). We investigated the expression levels of PCNA in $p57^{-/-}$ and wild-type embryos and observed that there were no differences (Figure 4a).

Zhang *et al*. (1998) reported that the mice with double mutations of both $p57^{Kip2}$ and $p27^{Kip1}$, and $p57^{Kip2}$-deficient mice, displayed abnormal development of placentae. Since they did not report the preterm delivery phenotype in their studies, this trophoblastic dysplasia might not be a reason for the preterm delivery that we observed in our $p57^{Kip2}$-deficient mice (Takahashi *et al*., 2000). This difference could be caused by differences in the deleted region; that is, in our mutant mice, the protein coding region of $p57^{Kip2}$ gene was completely eliminated, whereas for the mice reported by Zhang *et al*. (1997), and Yan *et al*. (1997), the coding region was partially deleted.

Most of the $p57^{-/+}$ female mice used in our study delivered offspring at 18.5 days post-coitum, 2 days earlier than usual (Takahashi *et al*., 2000). The trophoblastic dysplasia observed in our $p57^{-/-}$ and $p57^{+*/-}$ mouse embryos (Figure 2) may be the cause of preterm delivery. It has also been shown that $p57^{Kip2}$ expression is markedly reduced in patients with malignant trophoblastic neoplasms which can result in spontaneous abortions and preterm deliveries (Chilosi *et al*., 1998; N.Kanayama and K.Takahashi unpublished data). Together, these findings suggest that $p57^{Kip2}$ plays an important role in the regulation of trophoblastic proliferation in humans as well as in mice. Thus, $p57^{Kip2}$ may be important as a tumour suppressor during pregnancy.

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