J. Biochem. 127, 73–83 (2000)

Mice Lacking a CDK Inhibitor, $p57^{Kip2}$, Exhibit Skeletal Abnormalities and Growth Retardation

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Received August 2, 1999; accepted October 18, 1999

$p57^{Kip2}$, one of the cyclin-dependent kinase (CDK) inhibitors, has been suggested to be a tumor suppressor candidate. To elucidate its biological roles in mouse development and tumorigenesis, we created $p57^{Kip2}$-deficient mice. The $p57^{Kip2}$-deficient mice exhibited a cleft palate and defective bone formation resulting in severe dyspnea. Most of the $p57^{Kip2}$-deficient mice died within 24 h after birth, while about 10% of them survived beyond the weaning period. All of the surviving mice showed severe growth retardation. The males showed immaturity of the testes, prostate and seminal vesicles, and the females showed vaginal atresia, immaturity of the uterus, and an increased number of atretic follicles. Although Yan *et al.* and Zhang *et al.* have already reported $p57^{Kip2}$-deficient mice, they could not investigate the phenotypes of the surviving $p57^{Kip2}$-deficient mice. Also, most of the symptoms of Beckwith-Wiedemann syndrome could not be reproduced in the mutant mice. Embryonic fibroblasts prepared from $p57^{Kip2}$-deficient mice showed no differences in the proliferation rate and saturation density, suggesting that G1 arrest is largely independent of $p57^{Kip2}$ function. Our results suggest that $p57^{Kip2}$ plays a critical role in development, but do not support the hypothesis that the $p57^{Kip2}$ gene is a tumor-suppressor gene or is responsible for Beckwith-Wiedemann syndrome.

Key words: Beckwith-Wiedemann syndrome, CDK inhibitor, cell cycle, gene targeting, oncosuppressor gene.

Precise temporal and spatial control of the cell cycle is required for the development and homeostasis of higher eukaryotes. A series of cyclin-dependent kinases (CDKs) plays a central role in the regulation of cell cycle progression (*1, 2*). The activities of CDKs are regulated by several mechanisms including the binding of regulatory subunits (cyclins), phosphorylation and dephosphorylation of CDK itself, and the binding of CDK inhibitors (CKIs). CKIs are recognized as important proteins in cell cycle regulation. They are classified into two families, the Cip/Kip and Ink4 families. The Cip/Kip family members are $p21^{Cip1}$ (also called Waf1, Sdi1, or CAP20) (*3–7*), $p27^{Kip1}$ (*8, 9*), and $p57^{Kip2}$ (*10, 11*). They possess the ability to inhibit a variety of cyclin-CDK complexes and exhibit partial structural similarity. The Ink4 family members are $p16^{Ink4A}$ (*12*), $p15^{Ink4B}$ (*13*), $p18^{Ink4C}$ (*14, 15*), and $p19^{Ink4D}$ (*15, 16*), which are CDK4/CDK6-specific inhibitors. All CDK inhibitors induce G1 arrest when overexpressed in transfected cells (*17*).

$p57^{Kip2}$ can bind with a variety of cyclin-CDK complexes and inhibit their activities *in vitro*. $p57^{Kip2}$ exhibits homology with $p21^{Cip1}$ and $p27^{Kip1}$ in the N-terminal domain (CDK-binding/inhibiory domain), however, it is distinguishable from $p21^{Cip1}$ and $p27^{Kip1}$ in its unique domains, which are called the proline-rich domain and the acidic domain in mouse $p57^{Kip2}$, and a PAPA domain in human $p57^{Kip2}$. The overexpression of $p57^{Kip2}$ arrests cells in the G1 phase as that of $p27^{Kip1}$ does. In contrast to the widespread expression of $p21^{Cip1}$ and $p27^{Kip1}$, $p57^{Kip2}$, is expressed in a tissue-specific manner (*10, 11*). This gene is located within a cluster of imprinted genes, human chromosome 11p15.5 and mouse chromosome 7 (*18, 19*). In humans, this region is implicated in both sporadic cancers and Beckwith-Wiedemann syndrome, which is characterized by congenital malformations and organomegaly associated with an increased risk for development of childhood neoplasms, making $p57^{Kip2}$ a tumor suppressor candidate (*20*). Among patients with Beckwith-Wiedemann syndrome, mutations in the $p57^{Kip2}$ gene exist (*21*). With respect to human lung cancer, maternally biased 11p15 deletions occur (*22*). Not only several types of childhood tumors related to Beckwith-Wiedemann syndrome including Wilms' tumors, adrenocortical carcinoma and rhabdomyosarcoma, but also other types of cancer exhibit a specific loss of maternal 11p15 alleles (*23–25*). But in Wilms' tumor cell lines, there is no correlation between tumor suppression and $p57^{Kip2}$ expression (*26*). Although there is a wealth of information suggesting that $p57^{Kip2}$ may be associated with the tumor suppression, there has never been any direct evidence that $p57^{Kip2}$ functions as a tumor suppressive molecule. To clarify the physiological role of $p57^{Kip2}$ in cell cycle regulation during development, and to determine whether or not the loss of the

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$p57^{Kip2}$ gene results in a predisposition to cancer, we generated $p57^{Kip2}$-deficient mice. Our data indicate that $p57^{Kip2}$ plays an indispensable role in mouse development. However, we did not find a cancer predisposition in the mutant mice so far examined, suggesting that loss of $p57^{Kip2}$ is not simply responsible for tumorigenesis.

MATERIALS AND METHODS

Construction of a $p57^{Kip2}$ Targeting Vector and Production of Gene-Disrupted Mice—A 129/Sv mouse genomic library (Stratagene) was screened with mouse $p57^{Kip2}$ cDNA, kindly provided by Dr. Matsuoka, as a probe. From the 3×10^6 phages screened, a recombinant phage containing genomic DNA of the $p57^{Kip2}$ locus was isolated. The targeting vector was constructed by replacement of a *Xho*I–*Hin*dIII fragment containing the entire $p57^{Kip2}$ coding region with a PGK-neo-polyadenylate (poly[A]) cassette derived from pLNTK, which also had the PGK-tk-poly[A] cassette. The targeting vector contained 1.2 kb of homology 5′ and 8.1 kb 3′ of the neomycin-resistance marker, as shown in Fig. 1A. The maintenance, transfection and selection of E14 ES cells were carried out by means of established procedures (*27*). To screen homologous recombinant ES clones, we used cell lysates of G418- and gancyclovir-resistant clones as templates for PCR amplification with a $p57^{Kip2}$

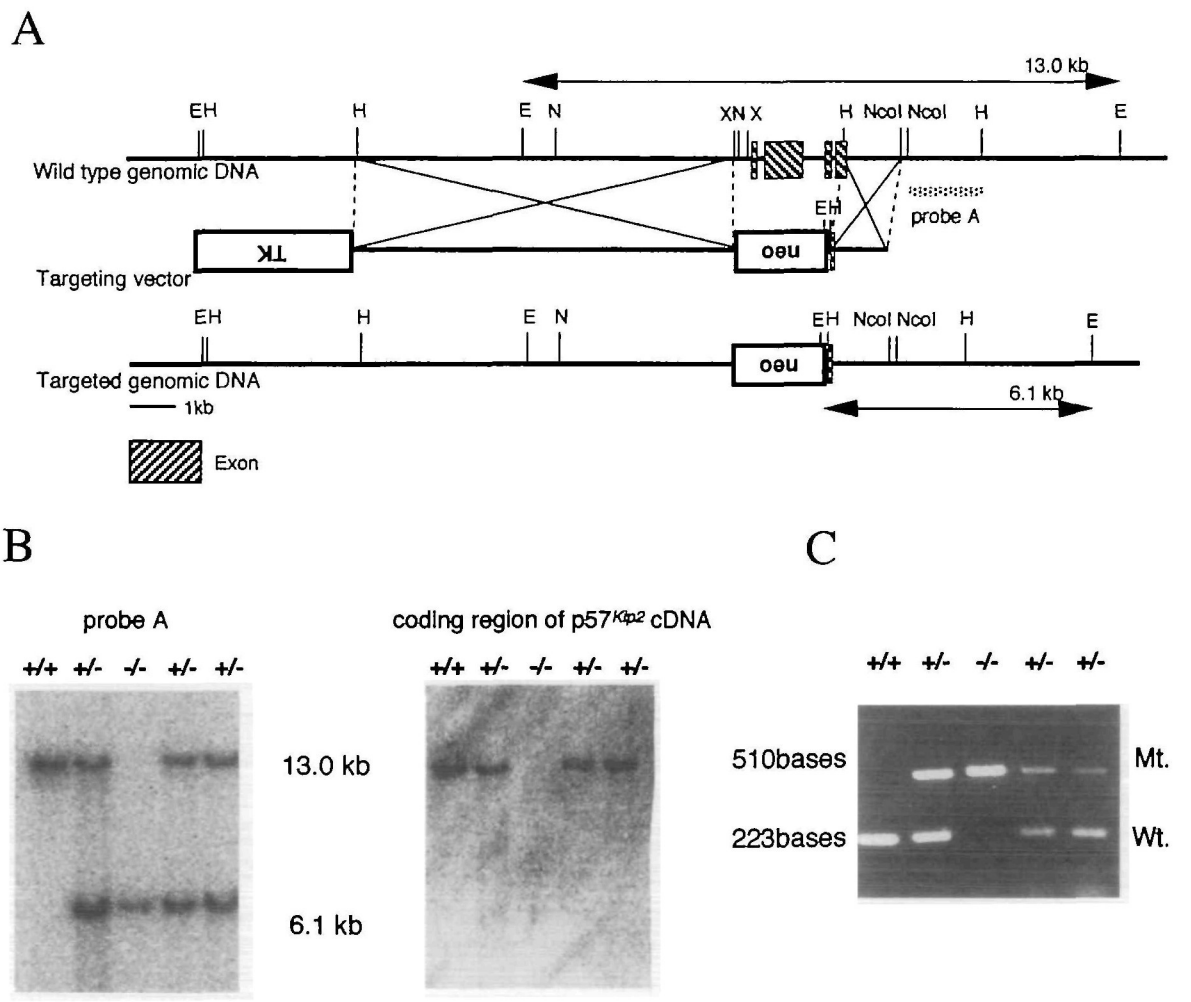

Fig. 1. **Targeted disruption of the mouse $p57^{Kip2}$ gene.** (A) Restriction map of the mouse $p57^{Kip2}$ gene, the targeting vector and the structure of the targeted genomic DNA following homologous recombination. The exons are denoted as closed boxes. The targeting vector contains a part of noncoding exon IV. The genomic fragment used as a probe for Southern blotting is shown as probe A. The abbreviations are as follows: neo, the neomycin transferase gene linked to the phosphoglycerate kinase (PGK) promoter; TK, the thymidine kinase gene derived from herpes simplex virus linked to the PGK promoter. Both neo and TK were placed in the reverse orientation relative to $p57^{Kip2}$ transcription. Restriction site abbreviations: E, *Eco*RI; H, *Hin*dIII; N, *Not*I; X, *Xho*I. The location of *Nco*I sites has not been fully determined. The expected sizes of *Eco*RI fragments that the probe are indicated. (B) Southern blot analysis of genomic DNA extracted from ES cells. The DNA was digested with and hybridized with probe A, and then the filter was stripped and rehybridized with the protein coding region of the mouse $p57^{Kip2}$ cDNA. The genotypes are presented above the lanes. (C) PCR amplification products of DNA extracted from mouse tails. The primers for the wild type allele were 5′-TGCACTGAGAGCGAGTAGAGATT-3′-(primer I) and 5′-AGCGGACCGATGGAAGAACTCTG-3′(primer II). For the mutant allele, primer I and 5′-GCGAAGGAACAAAGCTGCTATTG-3′(primer III) were used. The three primers were mixed together in the PCR reaction. The sizes of the wild type (223 bp) and mutant (510 bp) alleles were as indicated.

flanking primer (5′-AGCCTTGAACACCTGATAATGTCCA-3′) and a PGK promoter–specific primer (5′-ATGCTCCA-GACTGCCTTGGGAAAAGC-3′). To verify the results of our PCR screening, DNAs prepared from PCR-positive ES clones were digested with *Eco*RI, transferred to a nylon membrane (Pall), and then hybridized with the 1.4 kb *Nco*I–*Hin*dIII probe that flanked the 3′ homology region (Fig. 1A). The expected sizes for wild-type p57^{Kip2} and mutant p57^{Kip2} are 13.0 and 6.1 kb, respectively. The frequency of homologous recombinations was 13.5% of the double-resistant ES clones. ES cells heterozygous for the targeted mutation were microinjected into C57BL/6 blastocysts and then implanted into pseudopregnant ICR females. The resulting male chimeras were mated with female C57BL/6 mice. The germline transmission of injected ES cells was confirmed by inheritance of the agouti coat color in the F1 animals, and all agouti offspring were tested for the presence of the mutated p57^{Kip2} allele by PCR amplification with specific primer pairs for both the wild-type and mutant alleles, as shown in Fig. 1, and confirmed by Southern blot analysis under the same conditions as for the detection of homologous recombination events in the ES cells.

Histopathological Examinations—For morphological evaluation, whole bodies were fixed in Bouin's solution (10% formaldehyde, 0.7% picric acid, 5% acetic acid) for 24 h and changed in neutral pH formalin (3.7% formaldehyde, 45 mM Na_2HPO_4, 29 mM NaH_2PO_4). Dissected tissues were fixed in neutral pH formalin. Paraffin sections were prepared by standard procedures, and stained with hematoxylin and eosin. The results of histopathological analysis were examined by Bozo Research Center.

Skeleton Staining—Mice were eviscerated and then placed in acetone for 48 h. The carcasses were stained with a mixture of alcian blue and alizarin red (0.015% alcian blue, 0.005% alizarin red, 5% glacial acetic acid, 66.15% ethanol) for 24 h. The skeletons were rinsed in water for 3 h and then cleared in 1% KOH for 24 h. The skeletons were stored in a stock solution (35% ethanol, 50% glycerol).

Preparation of Rabbit Anti Mouse p57^{Kip2} Polyclonal Antisera—Polyclonal antisera against mouse p57^{Kip2} were raised in rabbits (New Zealand White) using GST-mouse p57^{Kip2}, which was expressed in *Escherichia coli* and purified with glutathione Sepharose. Immunoprecipitation with mouse p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, expressed by *in vitro* translation method, showed that the prepared antisera were specific for p57^{Kip2} protein detection.

Preparation of MEFs and Cell Culture—Mouse embryonic fibroblasts (MEFs) were obtained from 14.5 days postcoitus (dpc) embryos (*28*). To distinguish between the normal heterozygotes (p57–/+) and abnormal ones (p57+/–), embryos were obtained not only through mating between heterozygotes but also between the wild-type and heterozygotes. Determination of the saturated density and growth properties of unsynchronized MEFs at passage 3 was carried out as described (*29*). The proliferation properties of MEFs synchronized with serum-starved media were measured with a Cell proliferation kit II (Boehringer).

Immunoblot Analysis—MEFs from the indicated mouse genotypes were suspended at 3×10^7 cells/ml in Tween 20 lysis buffer (*28*). The protein concentrations of the total lysates were determined by the Bradford method (protein assay; Bio-Rad). The total lysates were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on a 8% gel for the detection of p57^{Kip2} or on a 12% gel for the detection of p27^{Kip1}, CDK2, and CDK4. They were transferred to Immobilon-P membranes (Millipore). The membranes were probed with either polyclonal antisera against mouse p57^{Kip2}, or polyclonal antibodies against p27^{Kip1} (Transduction Laboratories), p21^{Cip1} (Santa Cruz), CDK2 (Santa Cruz), and CDK4 (Santa Cruz). Proteins were visualized by ECL (Amersham).

Immuno Complex Kinase Assay for CDK2 and CDK4—Total lysates (100 μg) of embryonic fibroblasts prepared with Tween 20 lysis buffer were incubated with either rabbit anti-CDK2 antibodies (Santa Cruz) or rabbit anti-CDK4 antibodies (Santa Cruz) for 3 h on ice. The 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 50mM HEPES (pH 7.5), and then suspended in 30 μl of kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μCi of [γ-^{32}P] ATP [6,000 Ci/mmol; Amersham], 0.1 mM glutathione) with freshly prepared *E. coli* expressed GST-Rb proteins. The samples were incubated for 20 min at 30°C, denatured in SDS sample buffer, and then applied to a 10% SDS–polyacrylamide gel. The dried gels were examined with a BAS1000 image analyzer (Fuji Film) for 30 min.

RESULTS

p57^{Kip2} Gene Targeting—The murine p57^{Kip2} genome comprises four exons spanning about 2 kb. The coding region resides in exons 2 and 3. The targeting construct was designed to delete the entire coding region of the p57^{Kip2} gene (Fig. 1A). Electroporation of the linearized vector and G418/gancyclovir selection for homologous recombinants were carried out as described under "MATERIALS AND METHODS." One hundred ninety-two G418/gancyclovir double-resistant ES cell clones were picked up and screened for homologous recombination events by means of the poly-

TABLE I. **Genotypes of progeny derived from each of the crosses. Genotypes were determined by PCR amplification of DNA from the indicaed numbers of progeny at the indicated stages.**

♂ –/+ × ♀ –/+ (n = 367)

Age	+/+	–/+ or +/–	–/–
E14.5 (n = 37)	24.3	48.7	27.0
E16.5 (n = 31)	32.2	45.2	22.6
E17.5 (n = 31)	25.8	48.4	25.8
Newborn (n = 174)	25.4	52.8	21.8
4 weeks (n = 94)	46.8	51.1	2.1

♂ +/+ × ♀ –/+ (n = 256)

Age	+/+	+/–
E14.5 (n = 45)	48.9	51.1
E16.5 (n = 21)	57.1	42.9
E17.5 (n = 20)	50.0	50.0
Newsborn (n = 107)	55.1	44.9
4 weeks (n = 63)	93.7	6.3

♂ –/+ × ♀ –/+ (n = 185)

Age	+/+	–/+
E14.5 (n = 19)	47.4	52.6
E16.5 (n = 18)	44.4	55.6
E17.5 (n = 16)	50.0	50.0
Newsborn (n = 66)	45.5	54.5
4 weeks (n = 66)	45.5	54.6

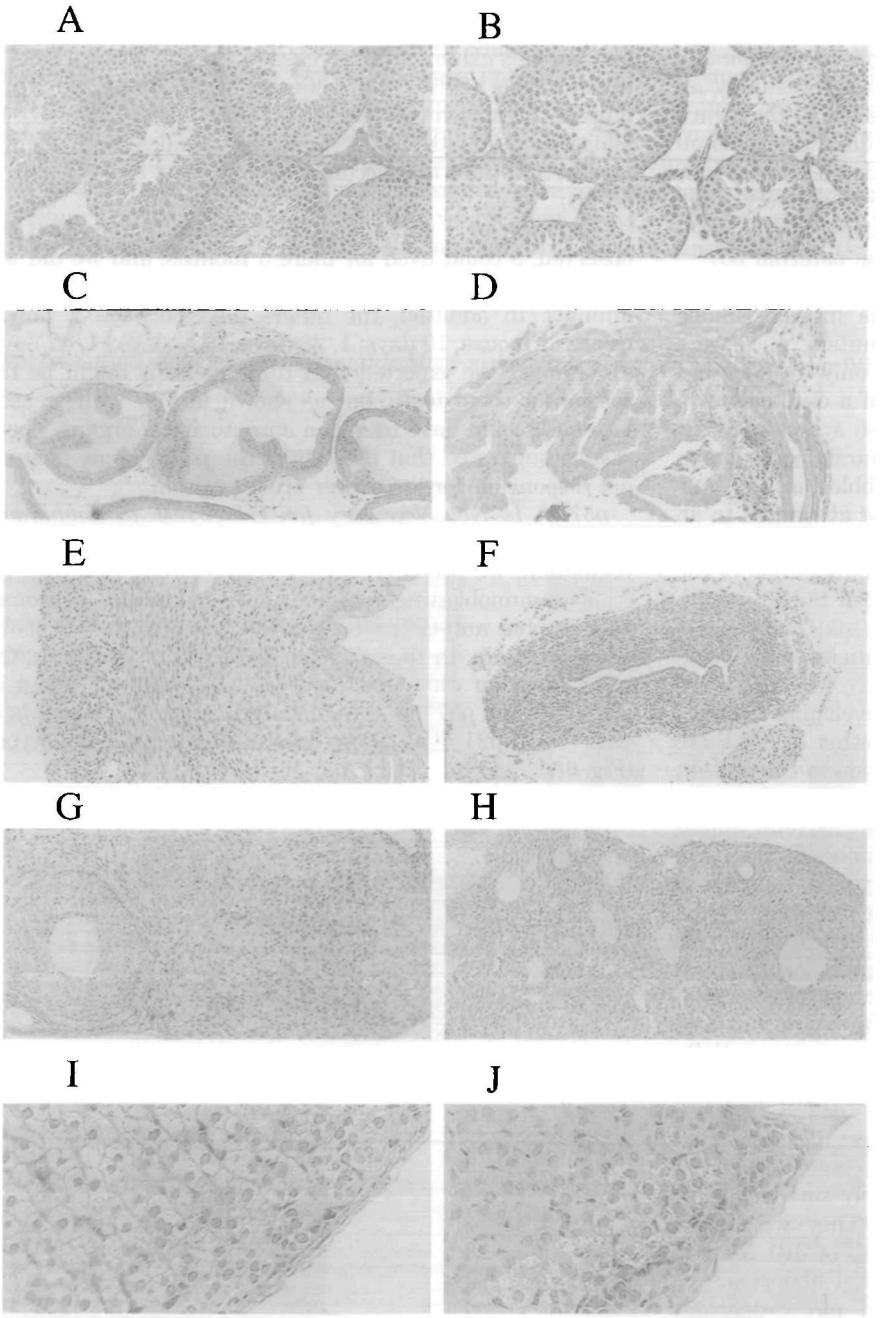

Fig. 4. **Histological analysis of surviving p57^{Kip2}-deficient mice.** Haematoxylin- and eosin-stained sections through the radius of a wild-type (A, C, E, G, I) and a 5 week-old p57^{Kip2}-deficient (B, D, F, H, J) mouse. Surviving p57^{Kip2}-deficient mice showed immaturity of several tissues. (A, B) testis; (C, D) prostate; (E,F) uterus; (G, H) ovary; (I, J) cortical cells of adrenal. Magnification: A–H, ×100; I and J, ×400.

The initial growth properties were examined in *in vitro* cultures after 3 passages of these MEFs. MEFs, regardless of whether they expressed the p57^{Kip2} protein or not, showed similar growth rates before reaching confluence. Both contact inhibition and the saturation density were independent of the expression of p57^{Kip2} (Fig. 6B). In addition, all MEFs showed growth arrest in serum-starved media for 72 h, but they began to proliferate after being transferred to the complete media. The p57^{Kip2} protein might have little influence on the proliferation properties of MEFs (Fig. 6C). We had expected that a p57^{Kip2} protein deficiency would induce an increase in the proliferation rate of MEFs through the inhibition of CDKs, however, the proliferation rate was not dependent on the expression of the p57^{Kip2} protein (Fig. 6).

Since we were interested in the relation between CDK kinase activity and p57^{Kip2} protein expression, we investigated the protein-expression levels and kinase activities of both CDK2 and CDK4 in embryonic fibroblasts. We used the *E. coli*–expressed glutathione S-transferase (GST) fusion protein with the C-terminal fragment of the Rb protein as the substrate for CDKs (*32*). We found that the expression levels of both CDK2 and CDK4 were similar among the four genotypes (p57+*/+, –/+, +*/–, and –/–), and that there was no difference in their activities, regardless of the genotypes (Fig. 7). We also recognized the inhibitory effects of the GST-p57^{Kip2} fusion protein on CDK kinase activities toward the GST-Rb C-terminal protein, in agree-

TABLE III. **Summary of phenotypes in 5 week $p57^{KIP2}$-deficient mice.**

Testis Immaturity	3/4 (75%)
Prostate Immaturity	3/4 (75%)
Seminal vesicle Immaturity	3/4 (75%)
Ovary Increased number of atretic follicles	5/5 (100%)
Uterus Immaturity	4/5 (80%)
Adrenal Decrease of lipid in cortical cells	8/9 (89%)
Spleen Underdevelopment of white pulp	6/9 (67%)

ment with Matsuoka *et al.* (*19*). In MEFs, similar to their proliferation properties, the kinase activities of CDK2 and CDK4 were not dependent on the expression level of the $p57^{Kip2}$ protein.

DISCUSSION

$p57^{Kip2}$ was identified as one of the universal inhibitors of CDK, as well as $p21^{Cip1}$ and $p27^{Kip1}$ (*10, 11, 17*). However, the biological differences between these three molecules remain unclear. To address this question, we and others have created mutant mice lacking $p21^{Cip1}$, $p27^{Kip1}$, or $p57^{Kip2}$ (*28, 29, 31, 32, 34–36,* this study). Surprisingl by the phenotypes of the gene-ablated mice were completed, distinct in spite of their structural and in vitro functional similarities. $p21^{Cip1}$-deficient mice seemed to develop normally, whereas $p27^{Kip1}$- and $p57^{Kip2}$-deficient mice exhibited abnormalities in late and early development, respectively. In contrast to our expectations, none of them showed an extensive predisposition to cancer, as seen in p53 or $p16^{Ink4A}$ gene–ablated mice (as discussed below) (*37, 38*).

There are unique properties that characterize the $p57^{Kip2}$ molecule: (i) its expression is tissue-specific (such as in the skeletal muscle, heart, central nervous system, cartilage, and placenta) (*10, 11, 31, 32*), (ii) the gene is located in the region which undergoes genomic imprinting, and the paternal allele is actually imprinted in humans and mice (*18, 19*), (iii) the gene is located next to that responsible for Beckwith-Wiedemann syndrome (*20*), and (iv) the protein possesses unique domains (proline-rich and acidic domains) with unknown functions, which are present in neither $p21^{Cip1}$ nor $p27^{Kip1}$ (*10, 11*). Thus, our interest in $p57^{Kip2}$-deficient mice was mainly focused on whether there are pathological defects in the tissues expressing the $p57^{Kip2}$ protein, whether there are any effects on the genomic imprinting of adjacent genes, whether the abnormalities seen in the Beckwith-Wiedemann syndrome can be recapitulated, and whether the cell-cycle control including the G1 checkpoint is affected.

Although other groups have already reported the generation of $p57^{Kip2}$-deficient mice (*31, 32*), the phenotypes of our mutant mice are clearly distinct in some points. First, not so many but a significant (about 10%) number of mutant mice survived for more than 5 months. Second, remarkable growth retardation was observed in all survivors. Third, vaginal atresia was present in all female survivors. Zhang *et al.* (1997) reported that no mice could survive beyond the neonatal period, while Yan *et al.* (1997) reported that about 10% of mutant mice could survive but did not exhibit any abnormalities. Fourth, $p57^{Kip2}$+/– females exhibited preterm delivery. These phenotypic differences among these three groups could be attributable to differences in the deleted region of the $p57^{Kip2}$ locus. We completely eliminated the protein-coding region of the $p57^{Kip2}$ gene, whereas other groups only partially deleted the coding region. In addition, the promoter driving the neomycin-resistance gene, and the transcriptional orientation of the neomycin-resistant gene might affect the phenotypes of the mutant mice. Moreover, differences in the genetic background and/or in environmental factors cannot be excluded.

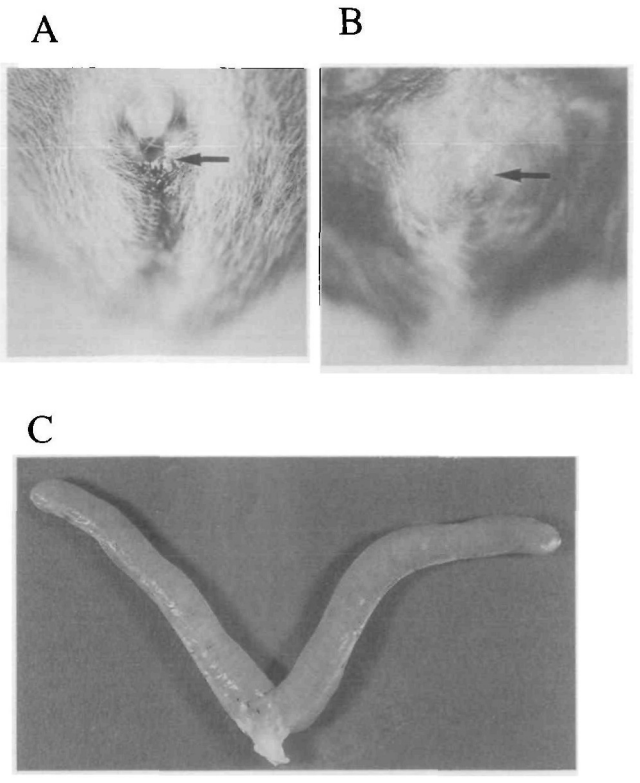

Fig. 5. **Vaginal atresia in the *long-lived* female $p57^{Kip2}$-deficient mice.** (A) Vaginal atresia in a surviving female $p57^{Kip2}$-deficient mouse (5 months; right). A wild-type control is shown on the left. Arrows indicate the opening of the vagina (left) and atresia (right). (B) Fluid accumulation in the uterus of a surviving female $p57^{Kip2}$-deficient mouse (5 months).

Furthermore, it is possible that the difference in the phenotypes is due to the disruption of unknown genes, promoters, enhancers, enhancers and/or silencers on the deleted region. Including $p57^{Kip2}$, genes located within this region are known to undergo genomic imprinting paternally or maternally. Although the imprinting mechanisms are largely unclear, it is likely that the expression of genes within this region could be affected by the deletion of the $p57^{Kip2}$-coding region and/or the insertion of the neomycin-resistant gene cassette. The remarkable growth retardation which was observed in all survivors had also been observed in IGF-2 gene targeting mice (*39*). The IGF-2 gene, whose maternally-inherited allele is transcriptionally repressed, is

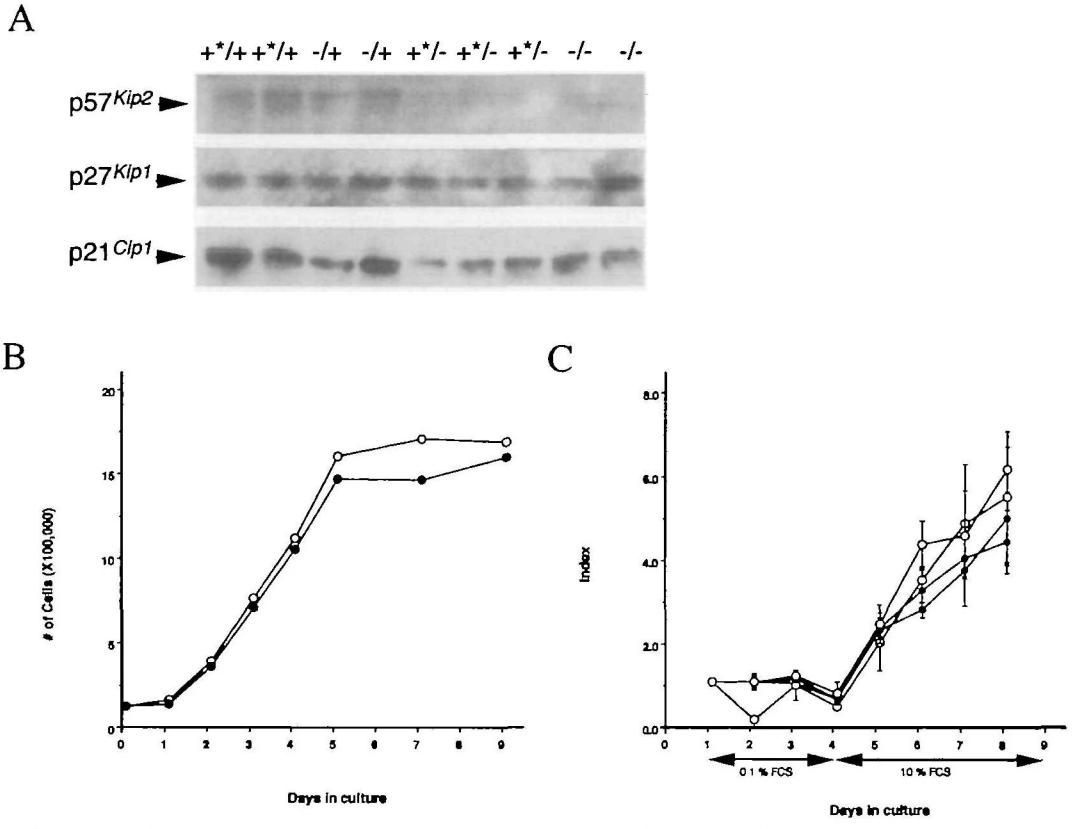

Fig. 6. **Disruption of p57Kip2 had no effect on the growth properties of MEFs.** (A) Immunoblot analysis of the $p57^{Kip2}$ protein in MEFs. Expression of the $p57^{Kip2}$ protein in MEFs was assessed by immunoblot analysis with anti-$p57^{Kip2}$ polyclonal antisera. The expression of both the $p27^{Kip1}$ and $p21^{Cip1}$ proteins was also assessed with their specific antibodies. (B) Saturation density and growth analysis of unsynchronized MEFs at passage 3. Cells (10^5) from wild-type (+*/+) and $p57^{Kip2}$ –/– mice were plated in replicate on 35 mm culture dishes on day 0, and the cells were counted on the indicated days. The cell count at each time represents the average for duplicate plates (*29*). $p57^{Kip2}$ +*/+, closed circles; $p57^{Kip2}$ –/–, open circles. (C) Proliferation curves for MEFs synchronized in serum-starved media. MEFs were seeded on 96-well plates in the complete media with 10% FCS at 5×10^3 cells/well. 24 h later, the media were changed to serum-starved media (0.1% FCS). After 72 h culture in the serum-starved media, the MEFs were exposed to the complete media again. The cells was determined as the specific absorbance at 490 nm with an XTT assay kit (Boehringer). An index was calculated as the comparative number with respect to the specific absorbance of the cells on day 1. Shown are the mean±standard deviations of the index obtained for triplicate cultures. $p57^{Kip2}$ +*/+, closed circles; $p57^{Kip2}$ –/–, open circles.

located 800 kb telomeric to the $p57^{Kip2}$ gene (*40*). The deletion of the H19 gene, which is located 80 kb telomeric to the IGF-2 gene, spoiled the imprinting of the IGF-2 gene (*41*). The control of imprinting is suggested to be controlled by DNA methylation, and in some cases, the methylation is regulated by an element which is located more than 1.5 Mb from the gene (*42*). From the results of immunoblot analysis of whole lysates derived from embryonic bodies (14 dpc), we could not recognize a difference in the expression level of IGF-2 among the genotypes (data not shown). But we could not disregard the possibility that the deletion of the $p57^{Kip2}$ gene might affect the mechanism regulating the expression of the IGF-2 gene, finally leading to growth retardation, as seen in IGF-2–deficient mice (*39*).

In our study, of $p57^{Kip2}$-deficient mice, about 90% died within 24 h after birth and the other 10% could survive with growth retardation. $p57^{Kip2}$-deficient neonates showed severe dyspnea resulting from skeletal abnormalities and died within 24 h after birth. Also on skeleton staining of surviving mice (5 weeks), we could not detect any abnormality except in their size (data not shown). This indicates that the progress of cartilage and bone formation determines the destiny of $p57^{Kip2}$-deficient mice. Most of the $p57^{Kip2}$-deficient survivors exhibited immaturity of germinal tissues including the testes, prostate, ovaries and uterus. $p57^{Kip2}$ might be necessary for the normal differentiation of germ cells, but this immaturity of organs may be due to the disruption of unknown genes.

The primary function of $p57^{Kip2}$ is postulated to be arrest of the cell cycle through inhibition of the CDK activity. However, the G1 arrest induced by contact inhibition, γ-irradiation (data not shown), and serum starvation seemed to be un affectedin $p57^{Kip2}$-deficient embryonic fibroblasts. Fibroblasts prepared from $p21^{Cip1}$ gene–ablated mice showed a partial defect in the G1 arrest (*29, 34*), whereas $p27^{Kip1}$-deficient fibroblasts exhibited normal G1 arrest (*28*). These data suggested that even though the $p57^{Kip2}$ protein is expressed in fibroblasts, it is not essential for G1 arrest, at least under our conditions, and other CKIs may compensate for the function of $p57^{Kip2}$. This hypothesis was supported by the fact that the CDK2 and CDK4 activities in $p57^{Kip2}$-deficient fibroblasts were comparable with those in

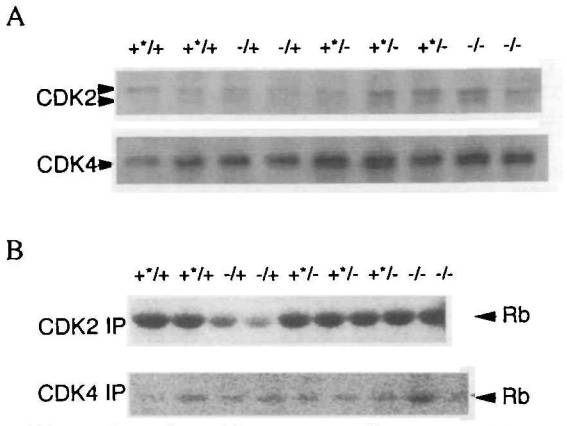

Fig. 7. **Disruption of p57Kip2 had no effect on the kinase activities of both CDK2 and CDK4 in MEFs.** (A) Expression levels of CDK2 and CDK4 in protein lysates of MEFs. The expression levels were assessed by immunoblot analysis, as described under "MATERIALS AND METHODS." The genotypes are presented above the lanes. CDK2 mRNA in mouse cells was translated as two protein isoforms of 32 and 40 kDa (*51*). (B) Kinase activities of CDK2 and CDK4 in protein lysates of MEFs. For the kinase assay, the GST-C terminal fragment of mouse pRb expressed by *E coli* was used as the substrate.

the wild-type fibroblasts.

It has been suggested that p57^{Kip2} is an oncosuppressor gene because of its ability to negatively regulate cell growth. Actually, in sporadic adult cancers and childhood neoplasms, genetic alterations were observed in the maternal allele of 11p15.5, where the p57^{Kip2} gene is located (*22–25*). However, we could not find any spontaneous tumors in the p57^{Kip2}-deficient mice during our 5-month observation of them. Yan *et al.* (1997) have also reported that no p57^{Kip2} null mice developed neoplasms. Some gene-ablated mice as to molecules related to cell cycle control, such as p53, p16^{Ink4a}, and E2F-1, showed a cancer predisposition (*37, 38, 43, 44*). Further observation of the p57^{Kip2} null mice and an *in vitro* assay involving p57^{Kip2}-deficient primary cells will be necessary to test late-onset tumorigenesis.

p57^{Kip2} is the candidate gene for Beckwith-Wiedemann syndrome, which is characterized by exophthalmos, macroglossia, gigantism and hypoglycemia. Because Beckwith-Wiedemann syndrome includes various patterns of hereditary transmission and phenotypes, it is most likely not caused by a simple mutation. The recent study by Elliot *et al.* (1996) revealed the frequencies of the complications in 76 patients with Beckwith-Wiedemann syndrome; macroglossia (97%), overweight (88%), abdominal wall defect (80%), ear deformity (76%), hypoglycemia (63%), facial naevus flammeous (62%), nephromegaly (59%), hemihypertrophy (24%), congenital heart defects (6.5%), polyductyly (4%), neoplasia (4%), development delay (4%), and cleft palate (2.5%). p57^{Kip2}-deficient mice did not exhibit most of the phenotypes of Beckwith-Wiedemann syndrome described above. These observations indicate that the lack of p57^{Kip2} expression is not the primary cause of Beckwith-Wiedemann syndrome. However, although the frequencies are low, the presence of developmental delay and a cleft palate in patients with Beckwith-Wiedemann syndrome suggested that expression of the p57^{Kip2} gene may be directly or indirectly affected in some patients.

Mice lacking p57^{Kip2} exhibited abnormal endochondral ossification and vaginal atresia. A defect in endochondral ossification was also seen in p107/p130 doubly-mutated mice (*45*), and vaginal atresia was observed in pRb+/–; p107–/– mutant mice (*46*). These phenotypic similarities imply a genetic link between p57^{Kip2} and pRb-related proteins (pRb, p107, and p130). It is of interest that the abnormalities seen in p27^{Kip1}-deficient mice are similar to those in mice with the Rb mutation, and in contrast to those in cyclin D1– and cyclin D2–deficient mice (*28, 47–50*). These observations suggest that p27^{Kip1} and p57^{Kip2} cooperatively regulate cell growth through pRb-related proteins.

In summary, the analysis of p57^{Kip2}-deficient mice revealed that the p57^{Kip2} gene is not related to Beckwith-Wiedemann syndrome, and that the p57^{Kip2} protein controls the progress of chondrocyte development. The phenotypes of the p57^{Kip2} deficient mice suggest that p57^{Kip2} may regulate the function of pRb-family molecules in both embryonic development and growth control. We did not observe any effect of p57^{Kip2} deficiency on MEFs properties, however, biochemical functions of p57^{Kip2} other than cell cycle regulation may exist. Further characterization of the biochemical basis of the observed defects should help elucidate the relevant pathways.

We are especially grateful to S. Matsuoka for kindly providing the mouse p57^{Kip2} cDNA. We also thank Ma. Satoh, H. Tateishi, N. Shinbori, Me. Satoh and M. Hirakawa of the Molecular Oncology Group at the Nippon Roche Research Center for the histopathological analysis, and M.Y. Nishikawa for critical reading of the manuscript. In addition, we thank K. Fukuchi, I. Negishi, Y. Shinkai, H. Matsushime, D.Y. Loh and M. Tomita for their valuable suggestions.

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