RESEARCH COMMUNICATION

Cooperation between the *Cdk* inhibitors *p27^{KIP1}* and *p57^{KIP2}* in the control of tissue growth and development

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Cell cycle exit is required for terminal differentiation of many cell types. The retinoblastoma protein *Rb* has been implicated both in cell cycle exit and differentiation in several tissues. *Rb* is negatively regulated by cyclin-dependent kinases (*Cdks*). The main effectors that down-regulate *Cdk* activity to activate *Rb* are not known in the lens or other tissues. In this study, using multiple mutant mice, we show that the *Cdk* inhibitors *p27^{KIP1}* and *p57^{KIP2}* function redundantly to control cell cycle exit and differentiation of lens fiber cells and placental trophoblasts. These studies demonstrate that *p27^{KIP1}* and *p57^{KIP2}* are critical terminal effectors of signal transduction pathways that control cell differentiation.

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Proper development of an organism requires an integration of cell cycle exit and differentiation pathways. Proliferation is positively regulated by cyclin-dependent kinases (*Cdks*), a family of highly regulated enzymes that link proliferative signals with mechanical aspects of cell duplication. Acting in opposition to *Cdks* are *Cdk* inhibitors, *CKIs*. Two families of *CKIs* have been identified. The *p21^{CIP1}* family contains *p21*, *p27^{KIP1}*, and *p57^{KIP2}* and inhibits all kinases involved in the G₁/S transition, whereas the *p16^{INK4a}* family, including *p15*, *p16*, *p18*, *p19*, inhibits *Cdk4* and *Cdk6* specifically (for review, see Harper and Elledge 1996). The biochemical activities and patterns of expression of *CKIs* during development (Matsuoka et al. 1995; Parker et al. 1995), together with data derived from in vitro differentiation systems (Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995), implicate these proteins as the primary effectors of signaling pathways that control cell cycle exit, an event that is critical for differentiation. However, of all the *CKIs*, only *p57* is required for embryonic develop-

ment (Deng et al. 1995; Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996; Serrano et al. 1996; Zhang et al. 1997; Yan et al. 1997). Loss of *p57* results in proliferative disorders in the lens and in cartilage, and defects in development of several tissues (Yan et al. 1997; Zhang et al. 1997).

Insights into the question of how cell cycle arrest and differentiation are integrated have come from the analysis of embryonic lens development. The lens is composed of differentiated lens fiber cells capped on the anterior surface by a layer of immature, mitotic epithelial cells (McAvoy 1980; Piatigorsky 1981). Formation of this structure involves spatially controlled proliferation and differentiation events that are dependent on the retinoblastoma (*Rb*) gene product, a critical target of *Cdks*. Loss of *Rb* leads to defects in cell cycle arrest and differentiation, as well as increased p53-dependent apoptosis (Morgenbesser et al. 1994; Liegeois et al. 1996). In other differentiation systems such as skeletal muscle, *Rb* appears to play a dual role; it acts as a growth suppressor facilitating G₁ arrest and is also required for activating the transcriptional program that brings about differentiation (for review, see Mulligan and Jacks 1998), possibly through physical association with critical transcription factors (Gu et al. 1993; Chen et al. 1996; Nead et al. 1998). Although cell cycle arrest and activation of differentiation processes may involve separable functions of *Rb* (Sellers et al. 1998), available evidence suggests that both of these functions require inhibition of *Cdks* (Rao et al. 1994; Skapek et al. 1995, 1996). However, the main effectors that down-regulate *Cdk* activity to activate *Rb* are not known in the lens or other tissues. In this study, using multiple mutant mice, we show that *p27^{KIP1}* and *p57^{KIP2}* function together in a redundant manner to control cell cycle exit and differentiation in the lens and the placenta.

Results and Discussion

Ocular lens development involves several steps. By embryonic day 11.5 (E11.5), a sphere of epithelial cells have formed the lens vesicle. At this stage, cells in the posterior region undergo cell cycle exit and begin to elongate toward the anterior wall. Three days later, elongation is complete and these differentiated fiber cells are capped on the anterior wall by a layer of immature epithelial cells. These cells proliferate and migrate to the equatorial zone where they exit the cell cycle and differentiate to form secondary lens fiber cells (for review, see McAvoy 1980; Piatigorsky 1981). Cells in the equatorial zone express high levels of *p57* (Fig. 1D; Zhang et al. 1997), and loss of *p57* allows these cells to continue to proliferate temporarily (Zhang et al. 1997). However, *p57*-deficient lens cells eventually undergo cell cycle exit and differentiate into lens fiber cells. These lenses are relatively normal but, in some genetic backgrounds, accumulate vacuoles indicative of incomplete lens fiber cell elongation and/or apoptosis. The ability of *p57*-deficient

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Figure 1. Expression of cell cycle regulatory genes during lens development. In situ hybridization was performed on transverse sections through the eye region of an E15.5 embryo using antisense probes as indicated. The arrow in *A* points to the pigmented epithelium (PE), which falsely stains positive for all probes because of the presence of pigmented granules in these cells. Arrows in *D* indicate cells in the equatorial zone of the lens that express high levels of *p57*. *p27* is expressed in the equatorial zone and in the retina (r). Scale bar, 200 μ m.

lens cells to differentiate, albeit with reduced kinetics, implies the existence of a second regulatory pathway controlling cell cycle exit in this tissue.

Cell cycle exit could be achieved by down-regulating cyclins or by inducing additional CKIs. To examine these possibilities, we performed in situ hybridization analysis to determine the transcriptional status of CKIs and D-type cyclins in the lens during differentiation. At day E15.5, all three D-type cyclin mRNAs are expressed in the lens, with *D2* showing the strongest expression (Fig. 1*A–C*). *D2* and *D3* also show mRNA expression in the posterior chamber, which contains primarily differentiated cells. *D1* and *D2* had been shown previously to be expressed at day E13.5 in cells of the anterior epithelia and equatorial zone, and *D2* expression was observed in the posterior chamber (Fromm and Overbeek 1996), suggesting that additional inhibitory signals are likely to be required to counteract their growth-promoting activities. Consistent with this notion, we observed expression of a second CKI, *p27*, in the same cells as *p57* in the equatorial zone (Fig. 1*E*) and in the posterior chamber of the lens. In contrast, *p21* transcripts were not detected in the lens (Fig. 1*F*).

Previous studies did not detect defects in lens development in *p27*-deficient mice (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). To determine whether the coincident expression of *p27* and *p57* was indicative of a redundant function, we generated mice mutant for both of these CKIs. Male *p27*^{+/−}*p57*^{+/+} and female *p27*^{+/−}*p57*^{+/p} (p denoting paternal origin of the mutant *p57* allele) mice were mated to enrich for double mutants. Because the *p57*^{KIP2} gene is imprinted, only the allele inherited from the mother (m denoting maternal origin) need be mutant to produce phenotypically null offspring (Zhang et al. 1997). No *p57*-deficient animals survived to the time of genotyping (10 days of age), irrespective of the status of *p27*, confirming our earlier find-

ing that *p57* is essential for neonatal survival (Zhang et al. 1997). Dead pups found in newborn litters were shown to have a *p57*^{+/−m} genotype. Although expected frequencies of mice with all possible genotypes were found in embryos harvested from E13.5 to E18.5 (data not shown), we observed a significant incidence of embryonic lethality in *p57* mutant embryos, as has been reported (Yan et al. 1997; Zhang et al. 1997). Interestingly, deletion of *p27* significantly increased the frequency of embryonic lethality by a factor of 2 when the viability of *p27*^{−/−}*p57*^{+/−m} animals (20% lethality) are compared to that of *p27*^{+/−}*p57*^{+/−m} animals (10% lethality). However, we were unable to make a valid comparison between the lethality of *p27*^{−/−}*p57*^{+/−m} (20% lethality) and *p57*^{+/−m} animals from our original report (10% lethality; Zhang et al. 1997), because of different genetic backgrounds (see Materials and Methods). Embryos died over a wide window between day E12 and E16.5, and embryos that were alive at the time of harvesting showed heterogeneous degrees of growth retardation indicative of intermediate penetrance, likely resulting from the nature of mixed genetic backgrounds among these animals (see Materials and Methods). Histopathological examination of mutant embryos failed to show defects in the cardiovascular system or erythropoiesis, common sources of embryonic lethality. However, defects were observed in the placenta of mutant animals, an organ critical for fetal development and survival. This defect will be discussed below.

Embryos from these crosses that were not affected by placental defects were examined for developmental phenotypes, including those reported previously for the *p57*^{+/−m} mutant. All affected tissues displayed phenotypes equivalent to those seen in *p57*-deficient embryos (Zhang et al. 1997), with the exception of the lens in which a profound defect was observed in the *p27*^{−/−}*p57*^{+/−m} double mutants. It should be noted that phenotypically, *p27*^{−/−} lenses were indistinguishable from wild-type lenses (data not shown) and *p27*^{+/−}*p57*^{+/−m} lenses were indistinguishable from lenses derived from *p57*^{+/−m} mice (Fig. 2*G–I*). However, *p57*^{+/−m} lens defects in the genetic background resulting from a cross with *p27*^{−/−} mice are slightly more severe than those we observed previously (Fig. 2*G–I*; Zhang et al. 1997).

Dramatic defects in lens development of double mutant mice are apparent as early as E13.5, a time at which posterior cells have normally already initiated elongation into primary lens fiber cells (Fig. 2*D*). Most striking is the finding that lens vesicles from double mutant mice are filled with nuclei as assessed histologically (Fig. 2*F*). Although cells adjacent to the posterior wall fail to elongate in the double mutant, this effect, albeit less dramatic, is also seen in this background in *p57*^{+/−m} mice (Fig. 2*E,F*). By E15.5, the posterior zone nuclear density has increased further in double mutants, and no nuclei are detected in wild-type or *p27*^{−/−} mutants, and far fewer nuclei are present in *p27*^{+/−}*p57*^{+/−m} or *p57*^{+/−m} lenses (Figs. 2*A–C* and 3*A–C*). The appearance of large numbers of nuclei in the lens fiber cell compartment is consistent with ectopic proliferation. To verify this sup-

Figure 2. *p57* and *p27* are required for lens development. (A–C) Hematoxylin and eosin-stained sections of E15.5 lenses from the indicated genotypes. Nuclei at the posterior edge of *p57*^{+/-m} (E) and *p27*^{-/-}*p57*^{+/-m} (F) lenses are indicated by the arrow. (G–I) Nuclei in lens sections derived from E14.5 mice revealed by DAPI staining. Scale bar, 200 μ m.

position directly, in situ BrdU incorporation assays were performed. The posterior chamber in E13.5 and E15.5 *p27*^{-/-}*p57*^{+/-m} lenses contained many more actively dividing cells than did those of *p57*^{+/-m} and *p27*^{+/-}*p57*^{+/-m} heterozygous animals (Fig. 3D–F; data not shown). Sections from *p27*^{-/-}*p57*^{+/-m} lenses at E15.5 displayed 66-fold more BrdU-positive cells than lenses from *p27*^{+/-}*p57*^{+/-} mice and 8-fold more than lenses from *p27*^{+/-}*p57*^{+/-m} mice, and similar values were observed at E13.5 (Fig. 3P). We also note that *p57* mutant lenses (regardless of *p27* status) are 15%–20% larger than wild-type lenses, and cataracts were apparent (Fig. 2A–C; data not shown).

The appearance of large vacuoles in the anterior chamber of *p57* mutant lens (irrespective of *p27* status) could be a result of the failure of fiber cells to elongate, or a consequence of cell death by both apoptosis and necrosis, or both. Lens fiber cell elongation is a hallmark of differentiation and requires proper temporal and spatial expression of lens crystallin proteins, expression patterns that serve as reliable markers of the lens differentiation program. Mice deficient for the transcription factor SOX1 display defects in the differentiation of lens fiber cells as indicated by the absence of induction of γ -crystallins, leading to incomplete elongation and large vacuoles in the lens (Nishiguchi et al. 1998). *p57*^{+/-m} lenses display substantially reduced levels of β - and γ -crystallins (Fig. 3H,K), compared to *p27*^{-/-} lenses (Fig. 3G,J), and crystallin expression is reduced to undetectable levels when combined with *p27* deficiency (Fig. 3I,L). Control experiments (not shown) indicate that the staining seen in the region of the vacuoles in the anterior of the chamber is due to nonspecific interactions (edge effect) of the secondary antibody used and does not reflect crystallin expression. These data indicate that *p57* and *p27* are required for proper lens fiber cell differentia-

tion and elongation. To examine cell death, TUNEL assays were performed on E15.5 lenses. Apoptotic cells were detected in the posterior chamber of *p27*^{+/-}*p57*^{+/-m} and *p27*^{-/-}*p57*^{+/-m} mutant lenses, typically one apoptotic cell per 0.2-mm² cross section, but no apoptotic cells were detected in *p27*^{-/-} lenses in this region (Fig. 3M–O). Because there are ~100 cross sections per lens, there are ~100 apoptotic cells per lens compared to zero in a *p27* null lens. Histological evidence of necrosis was also found and was most pronounced in regions immediately adjacent to vacuoles (data not shown). Thus, cell

Figure 3. Overproliferation of lens fiber cells in *p27/p57* double mutants leads to compromised differentiation and increased apoptosis. (A–C) Nuclei of lens sections derived from E15.5 embryos revealed by DAPI staining. (D–F) BrdU incorporation assays demonstrate defects in cell cycle exit in *p27*^{-/-}*p57*^{+/-m} lenses. (G–L) Immunofluorescence staining of β - and γ -crystallins demonstrates reduced expression of these two differentiation markers in the lens of *p57*^{+/-m} mice (H,K) and the absence of expression in the lens of *p27*^{-/-}*p57*^{+/-m} mice (I, L). (M–O) TUNEL assays detect apoptotic cells (arrows) in both *p57*^{+/-m} and *p27*^{-/-}*p57*^{+/-m} lenses. (P) Quantitation of BrdU incorporation assays at E13.5 and E15.5. BrdU-positive nuclei from a total of six sections for each genotype were determined, and the average is shown in the histogram along with the s.d.. Scale bars in C, F, I, and L, 200 μ m; O, 50 μ m.

death may also contribute to vacuoles in *p57*-deficient lenses.

Developmental defects in the placenta were also observed in *p27^{-/-}p57^{+/-m}* mice. Of the several types of placentas, mice primarily have hemochorial placenta where maternal blood is no longer contained in blood vessels but is in direct contact with fetal trophoblasts that also embed fetal capillaries in the labyrinth zone. In placentas derived from *p57^{+/-m}* single or *p27^{-/-}p57^{+/-m}* double mutants, the labyrinth zone was less vascularized and contained more trophoblasts than those from wild-type or *p27^{-/-}* mice (Fig. 4A, cf. a and b). The diameter of most mutant fetal capillaries was reduced to the size of a single fetal red blood cell, leading to the appearance of less vascularization. Normal placentas contain numerous open spaces (the fetal capillary and maternal blood sinus) that are replaced with trophoblasts in the mutant. We have found that this phenotype varies considerably, ranging from very little vascularization (Fig. 4A,b) to almost normal in those animals who survived to term (not shown), consistent with the variability in timing and rates of embryonic lethality. The degree of placental impairment correlates with size of the embryo, with more developmentally defective placentas containing smaller embryos.

In addition to reduced vascularization, placentas from *p57* mutant mice, regardless of the status of *p27*, contain areas that are marked by hyaline membranes in the labyrinth zone (Fig. 4A,c; data not shown). Necrosis was observed in these areas and is likely to be due to blockade of the blood supply by hyaline membranes. Hyaline membranes are formed in response to endothelium damage, as has been observed in the respiratory distress syndrome caused by capillary or alveolar epithelium damage (Kobizk and Schoen 1994). Given the biochemical functions of *p27* and *p57* and their roles in other tissues, we suspected that the absence of these CKIs might alter the differentiation of trophoblasts in the labyrinth zone, allowing them to proliferate inappropriately. This would result in limited space available for the fetal capillaries and maternal blood sinus, possibly leading to blood vessel damage and the formation of hyaline membranes. BrdU incorporation assays demonstrated increased proliferation in *p27^{-/-}p57^{+/-m}* mutant placentas (Fig. 4C, cf. a and b). These assays were performed on a litter harvested at E18.5 when the normal placenta had already ceased proliferation to observe more easily the proliferation defects due to inhibitor loss. The fraction of BrdU-positive cells was greatly increased in placentas from *p27^{-/-}p57^{+/-m}* mice relative to *p27^{+/-}* and *p27^{-/-}* placentas (25- and 10-fold, respectively) and was significantly larger (4-fold) than that found with *p27^{+/-}p57^{+/-m}* placentas (Fig. 4C). There was considerable heterogeneity in the placental phenotypes of mutant animals. As shown in Table 1, a significant percentage of the *p27^{-/-}p57^{+/-m}* embryos escape embryonic lethality, and placentas from these animals appear to be much less defective than those shown here. Furthermore, although the additional loss of *p27* increases proliferation rates, it does not significantly exacerbate the histological defects observed in *p57* mutant placentas. It does, however, change the penetrance of the placental failure phenotype, making the placenta twice as likely to fail. *p57* is highly expressed in the labyrinth zone but not the adjacent spongiotrophoblast zone (Fig. 4B,a). All of the placental defects are observed exclusively in the labyrinth zone, whereas other aspects of the placentas from these mutant mice are normal (not shown). *p27* is expressed both in labyrinth and spongiotrophoblast zone (Fig. 4B,c). This concordance of expression suggests that the defects observed are cell autonomous and indicates that *p27* can provide some compensatory function in the labyrinth zone in the absence of *p57*. Thus, both *p27* and *p57* are expressed in the tissue found defective in mutant embryos, suggesting that the phenotype is very likely to be cell autonomous.

Figure 4. *p57* and *p27* are required for the proper development of the mouse hemochorial placenta. (A) Hematoxylin and eosin-stained E12.5 placenta sections from *p27^{+/-}* (a), *p57^{+/-m}* (b), and *p27^{+/-}p57^{+/-m}* (c) mice. Arrows in c indicate hyaline membranes. (B) Expression of *p57* (a) and *p27* (c) in E12.5 placenta as detected by in situ hybridization. The specificity of the *p57* probe is demonstrated through the absence of signal in a placental section from a *p57^{+/-m}* mouse (b). (C) BrdU incorporation assays reveal overproliferation in the labyrinth zone of *p27^{-/-}p57^{+/-m}* placentas, as compared with *p27^{+/-}*, *p27^{-/-}*, or *p27^{+/-}p57^{+/-m}* placentas. Placentas were harvested at E18.5. Quantitation of BrdU assays on embryos collected from a single litter are presented in c. Error bars represent the s.d. (Lz) Labyrinth zone; stz, spongiotrophoblasts zone. Scale bars, 200 μ m.

rinth zone (Fig. 4A,c; data not shown). Necrosis was observed in these areas and is likely to be due to blockade of the blood supply by hyaline membranes. Hyaline membranes are formed in response to endothelium damage, as has been observed in the respiratory distress syndrome caused by capillary or alveolar epithelium damage (Kobizk and Schoen 1994). Given the biochemical functions of *p27* and *p57* and their roles in other tissues, we suspected that the absence of these CKIs might alter the differentiation of trophoblasts in the labyrinth zone, allowing them to proliferate inappropriately. This would result in limited space available for the fetal capillaries and maternal blood sinus, possibly leading to blood vessel damage and the formation of hyaline membranes. BrdU incorporation assays demonstrated increased proliferation in *p27^{-/-}p57^{+/-m}* mutant placentas (Fig. 4C, cf. a and b). These assays were performed on a litter harvested at E18.5 when the normal placenta had already ceased proliferation to observe more easily the proliferation defects due to inhibitor loss. The fraction of BrdU-positive cells was greatly increased in placentas from *p27^{-/-}p57^{+/-m}* mice relative to *p27^{+/-}* and *p27^{-/-}* placentas (25- and 10-fold, respectively) and was significantly larger (4-fold) than that found with *p27^{+/-}p57^{+/-m}* placentas (Fig. 4C). There was considerable heterogeneity in the placental phenotypes of mutant animals. As shown in Table 1, a significant percentage of the *p27^{-/-}p57^{+/-m}* embryos escape embryonic lethality, and placentas from these animals appear to be much less defective than those shown here. Furthermore, although the additional loss of *p27* increases proliferation rates, it does not significantly exacerbate the histological defects observed in *p57* mutant placentas. It does, however, change the penetrance of the placental failure phenotype, making the placenta twice as likely to fail. *p57* is highly expressed in the labyrinth zone but not the adjacent spongiotrophoblast zone (Fig. 4B,a). All of the placental defects are observed exclusively in the labyrinth zone, whereas other aspects of the placentas from these mutant mice are normal (not shown). *p27* is expressed both in labyrinth and spongiotrophoblast zone (Fig. 4B,c). This concordance of expression suggests that the defects observed are cell autonomous and indicates that *p27* can provide some compensatory function in the labyrinth zone in the absence of *p57*. Thus, both *p27* and *p57* are expressed in the tissue found defective in mutant embryos, suggesting that the phenotype is very likely to be cell autonomous.

Analysis of mice lacking both *p27* and *p57* has revealed that these two CKIs cooperate to control cell cycle exit and differentiation in both the lens and placenta. In the lens, *p57* plays a role in cell cycle arrest in both posterior lens vesicle cells during primary differentiation and equatorial cells during secondary differentiation. *p57* levels increase dramatically in equatorial cells at the time of cell cycle exit and these cells proliferate inappropriately, albeit temporarily, in the absence of *p57* (this work; Zhang et al. 1997). In contrast, *p27* is normally not required for lens development but contributes significantly to cell cycle arrest and differentiation in the absence of *p57*. Although these inhibitors are expressed

at the highest levels in the equatorial zone, they are also expressed at low levels throughout the lens. Persistent expression of these two *CKIs* is necessitated by the continued presence of D-type cyclins in the developing lens (Fig. 1A–C), which also helps explain the unscheduled S-phase entry in the absence of these inhibitors. The inability of lens fiber cells to undergo cell cycle arrest leads to defects in differentiation, including elongation and β/γ -crystallin expression, which are more severe in *p57/p27* double mutants than in *p57* single mutants.

The phenotypes observed in *p27/p57* mutant lenses are reminiscent of those seen in *Rb*-deficient lenses (Morgenbesser et al. 1994), consistent with the biochemical roles of *CKIs* as activators of *Rb*. Because hypophosphorylated *Rb* plays a critical role in differentiation, it is likely that the inability of lens fiber cells to differentiate in *p27/p57* mutants reflects increased *Rb* phosphorylation and inhibition of its differentiation-promoting function. However, two significant differences exist between the phenotypes of the *Rb* versus *p27/p57* double mutants. First, the extent of overproliferation as assessed by BrdU incorporation appears to be significantly greater in *p27/p57* mutants than in *Rb* mutants. This may reflect the fact that these two *CKIs* function not only upstream of *Rb* by blocking cyclin D/*Cdk4* activity but also function downstream of *Rb* by blocking cyclin E/*Cdk2*-mediated S-phase entry. Alternatively, the increase in *Cdk* activity due to *CKI* loss may result in inactivation of additional *Rb*-family members such as p130 and p107, thereby producing a more severe proliferation defect than *Rb* loss alone. Thus, proliferation of lens fiber cells lacking *Rb* may be limited because of the action of *p27* and *p57* on *Cdks*. The second major difference is that the rates of apoptosis in *CKI*-deficient lenses are much lower than those in *Rb*-deficient lenses and are similar to the rates seen in *Rb/p53* double mutant lenses. *Rb* is required to establish the transcriptional program that brings about differentiation of multiple cell types but has also been shown to inhibit apoptosis during myoblast differentiation (Wang et al. 1997) and in other situations (for review, see Wang 1997). Thus, low rates of apoptosis in *p27/p57*-mutant lenses may reflect an antiapoptotic role for *Rb*. If the absence of *p27* and *p57* result in the inactivation of *Rb* to such an extent that it phenocopies the differentiation defect of the *Rb* null mutant lenses, why the difference in apoptosis rates? There are several plausible explanations for this difference. First, *Rb* could have an antiapoptotic function that is not regulated by *Cdk* phosphorylation and therefore would not be altered by *CKI* loss. Second, even in the absence of the *CKIs*, there may be residual *Rb* activity such that apoptosis-inhibiting functions of *Rb* are largely intact. Even in the absence of *CKIs*, there is likely to be residual regulation of *Rb* if *Cdk* activity is still cyclical. In contrast, an *Rb* null mutant cell would constitutively derepress all *Rb*-regulated genes such as E2F1, an apoptosis-inducing gene (Qin et al. 1994; Shan and Lee 1994; Kowalik et al. 1995) and might display a more severe phenotype for this reason. Third, it is also possible that *CKI* mutant cells have higher *Cdk* activity

levels and these act to prematurely inactivate E2F1 function (Dynlacht et al. 1994; Krek et al. 1994), thereby balancing the apoptotic-inducing consequences of inactivating *Rb*. The fact that the apoptosis rates of the *p27/p57* double mutants are similar to the rates observed in the *Rb/p53* double mutant mice (Morgenbesser et al. 1994) is consistent with interfering with E2F1 function because apoptosis caused by *Rb* loss is partially mediated by E2F1 (T. Jacks, pers. comm.) and E2F1-mediated apoptosis is p53-dependent (Qin et al. 1994; DeGregori et al. 1997).

CKIs are the ultimate effectors of signal transduction pathway intended to bring about cell cycle arrest, and the patterns of expression during embryonic development suggest that particular *CKIs* play important roles in terminal differentiation in a tissue-specific manner. However, the fact that mice lacking single *CKIs* display surprisingly few developmental phenotypes has brought into question the essential nature of *CKIs* for cell cycle arrest and differentiation. Our results demonstrate that two *CKIs*, *p57* and *p27*, cooperate to control proliferation and differentiation in multiple tissues and reiterate the critical importance of *CKIs* to cell cycle control during development.

Materials and methods

Genotypic analysis

Mice deficient for *p57* and *p27* (Δ -51 allele) have been described (Kiyokawa et al. 1996; Zhang et al. 1997). The original *p57* knockout was made in AB1 ES cells derived from a substrain of the 129 mouse, 129SvEvBrd-Hprt^{b-m2}, whereas the *p27* knockout was in the ES line CJ7, derived from another substrain of 129 mouse, 129Sv. These two 129 substrains are quite different according to Simpson et al. (1997). Thus, the original *p57* knockout resides in a background hybrid between C57BL/6 and 129SvEvBrd-Hprt^{b-m2}, and the current cross produced double mutants in a mixed background of C57BL/6, 129Sv, and 129SvEvBrd-Hprt^{b-m2}. We have developed PCR protocols to identify wild-type and disrupted alleles of *p27* and *p57*, using a set of three primers for each gene. For *p27*, the sequences of the primers are primer 1, ACGT-GAGAGTGCTAACGG; primer 2, AGTGCTTCTCCAAGTCCC; and primer 3, GCGAGGATCTCGTCGTGAC. For *p57*, the sequences of the primers are primer 1, CGTCCACAGGCCGAGTGC; primer 2, GCTGCGGAGGTACACGTCG; and primer 3, GCGAGGATCTCGTCGTGAC. Detailed protocols are available upon request.

Phenotypic analysis

Embryos were processed using standard histological procedures. β - and γ -crystallins were detected using polyclonal antibodies provided by K. Mahon (Baylor College of Medicine, Houston, TX) and visualized with FITC-conjugated secondary antibody (Amersham). For cell proliferation assays, pregnant mice were injected with BrdU (0.1 mg/gram body weight) 2 hr prior to delivery by cesarean section. S-phase cells were visualized using an anti-BrdU monoclonal antibody (Dako) in conjunction with an FITC-conjugated secondary antibody (Amersham). In situ hybridization was performed as described (Matsuoka et al. 1995; Parker et al. 1995). Probes for D-type cyclins were provided by C. Sherr (St. Jude Children's Research Hospital, Memphis, TN). Apoptotic cells were detected with a kit from Trevigene, and assays were performed as recommended by the manufacturer.

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