

# Lactation defect in mice lacking the helix–loop–helix inhibitor *Id2*

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**Id proteins are thought to be negative regulators of cell differentiation and positive regulators of cell proliferation. Mammary glands of *Id2*<sup>-/-</sup> female mice reveal severely impaired lobulo-alveolar development during pregnancy. *Id2*<sup>-/-</sup> mammary epithelia show no precocious maturation, but instead exhibit intrinsic defects in both cell proliferation and cell survival, implying that the role of *Id2* in pregnant mammary epithelia is mainly stimulation of cell proliferation and support of cell viability. Expression studies of genes required for mammary gland development suggest *Id2* to be a downstream or parallel factor of these genes. A decrease in the DNA binding activity of *Stat5* was also observed in *Id2*<sup>-/-</sup> mammary glands at 7 days post-coitus. Our results indicate an indispensable role of *Id2* in pregnant mammary glands.**

**Keywords:** apoptosis/cell proliferation/HLH inhibitor/*Id2*/mammary gland

## Introduction

The mammary gland is a unique organ, terminal differentiation of which is achieved during pregnancy under the influence of various hormones, including estrogen, progesterone and prolactin, and epithelial–mesenchymal interaction (Neville and Daniel, 1987). Dramatic changes in the mammary epithelia during pregnancy, characterized by expansion of the lobulo-alveolar compartment and maturation of epithelial cells for milk secretion, provides a good experimental model system to study the mechanisms that regulate cell proliferation and differentiation *in vivo* (Neville and Daniel, 1987).

Mammary gland development consists of multiple steps; establishment of the anlage during embryogenesis, ductal elongation and branching in puberty and lobulo-alveolar expansion in pregnancy (Neville and Daniel, 1987). Recent studies using gene targeting experiments have revealed some of the genetic components involved in each step of mammary gland development in mice (reviewed in Hennighausen and Robinson, 1998). Parathyroid hormone-related peptide is required to establish the mammary gland anlage during development (Wysolmerski *et al.*, 1998). The estrogen receptor is

essential for ductal elongation (Korach *et al.*, 1996) and genes required for lobulo-alveolar development include those encoding prolactin, prolactin receptor, *Stat5a*, progesterone receptor, *SRC-1*, cyclin D1, *C/EBPβ* and *A-myb* (Fantl *et al.*, 1995, 1999; Lydon *et al.*, 1995; Sicinski *et al.*, 1995; Horseman *et al.*, 1997; Humphreys *et al.*, 1997; Liu *et al.*, 1997; Ormandy *et al.*, 1997; Toscani *et al.*, 1997; Briskin *et al.*, 1998, 1999; X.Liu *et al.*, 1998; Robinson *et al.*, 1998; Seagroves *et al.*, 1998; Teglund *et al.*, 1998; Xu *et al.*, 1998). Although each of these genes has been shown to play an essential role in mammary gland development, their relationships are still largely unknown.

Transcription factors bearing a basic helix–loop–helix (bHLH) motif, exemplified by *MyoD*, are crucial for various cell differentiation processes during development in multicellular organisms (Weintraub *et al.*, 1991; Massari *et al.*, 2000). These factors are divided into two classes according to their expression patterns; ubiquitous bHLH factors (also known as E proteins) such as the *E2A* gene products *E12* and *E47*, and tissue-specific bHLH factors such as *MyoD* and *neurogenin* (Weintraub *et al.*, 1991; Massari and Murre, 2000). *Id* proteins, inhibitors of DNA binding/differentiation, which are characterized by their lack of the basic DNA-binding domain but retention of the HLH dimerization motif, inhibit the functions of these bHLH transcription factors in a dominant-negative manner by suppressing their heterodimerization partners through the HLH domains (Benezra *et al.*, 1990; Norton *et al.*, 1998). They have also been shown to stimulate the G<sub>1</sub>/S transition in the cell cycle in several cell culture systems, implying their roles in cell proliferation (reviewed in Norton *et al.*, 1998). Thus, *Id* proteins are considered to be positive regulators of cell proliferation as well as negative regulators of cell differentiation. Four related proteins have been identified in mammals so far (Benezra *et al.*, 1990; Christy *et al.*, 1991; Sun *et al.*, 1991; Riechmann *et al.*, 1994) and several lines of emerging evidence have demonstrated their involvement in various cell differentiation processes and cellular functions *in vivo*. We previously reported that mice deficient in *Id2* are devoid of lymph nodes and Peyer's patches due to lack of a cell population essential for the generation of peripheral lymphoid organs (Yokota *et al.*, 1999). In addition, *Id2* null mutant mice display disturbed differentiation of natural killer cells (Yokota *et al.*, 1999). In mice lacking *Id3*, the immune response and B-cell proliferation are impaired (Pan *et al.*, 1999). Moreover, *Id1* and *Id3* double knockout mice exhibit precocious maturation of neurons with elevated expression of cyclin-dependent kinase inhibitors and vascular malformation in the central nervous system (Lyden *et al.*, 1999).

Here we report that *Id2* is indispensable for mammary gland development during pregnancy. The observed defect is intrinsic to mammary epithelial cells. Our results

suggest that the function of *Id2* in pregnant mammary glands is to stimulate cell cycle progression and to support cell survival.

## Results

During the course of colony expansion of *Id2*-deficient mice we noticed that pups born to *Id2*<sup>-/-</sup> mothers died within 2 days after birth. The deaths were presumably due to dehydration, as examination revealed the absence of milk in their stomachs. This phenomenon was specific to homozygosity of the mothers and was not related to the genotype of the pups. Since *Id2*<sup>-/-</sup> mothers showed normal nursing behavior, a lactation defect was suspected. In cross-fostering experiments between *Id2*<sup>+/-</sup> and *Id2*<sup>-/-</sup> mothers none of 24 pups survived with *Id2*<sup>-/-</sup> mothers, while 34 out of 36 pups nursed by *Id2*<sup>+/-</sup> mothers did, irrespective of the genotype of the fostered pups (genotype ratios +/+:+/-:-/- were 0:22:14 and 1:11:12 for pups fostered by *Id2*<sup>+/-</sup> and *Id2*<sup>-/-</sup> mothers, respectively). The results confirmed that *Id2*<sup>-/-</sup> mothers have a lactation defect.

Whole mount analyses of the mammary glands demonstrated that *Id2*<sup>-/-</sup> virgin female mice have normal ductal trees, similar to those of heterozygous mice, indicating that normal development occurs before pregnancy (Figure 1A). Examination on the day of delivery, however, revealed that the glands of *Id2*<sup>-/-</sup> females were poor in lobulo-alveolar tissues and remained immature, comparable with those of *Id2*<sup>+/-</sup> mice at ~7 days post-coitus (d.p.c.), although side branching of mammary ducts was observed (Figure 1A, upper, and B). This failure of lobulo-alveolar expansion in *Id2* null mammary glands was also confirmed by histological analyses of sectioned specimens (Figure 1A, middle and lower). To evaluate the maturation status of the *Id2*<sup>-/-</sup> mammary glands, gene expression of milk proteins was investigated by northern blotting. Milk protein genes such as *WDM1*,  $\beta$ -casein,  $\alpha$ -lactalbumin and *WAP* start to be expressed sequentially in the mammary glands during pregnancy (Robinson *et al.*, 1995). In contrast to the abundant expression of all of these genes in *Id2*<sup>+/-</sup> mammary glands, only the early markers, *WDM1* and  $\beta$ -casein, were weakly detected in *Id2*<sup>-/-</sup> mammary glands on the day of delivery (Figure 1C), indicating poor differentiation. These results demonstrated that *Id2* is dispensable prior to pregnancy but essential during pregnancy for mammary gland development.

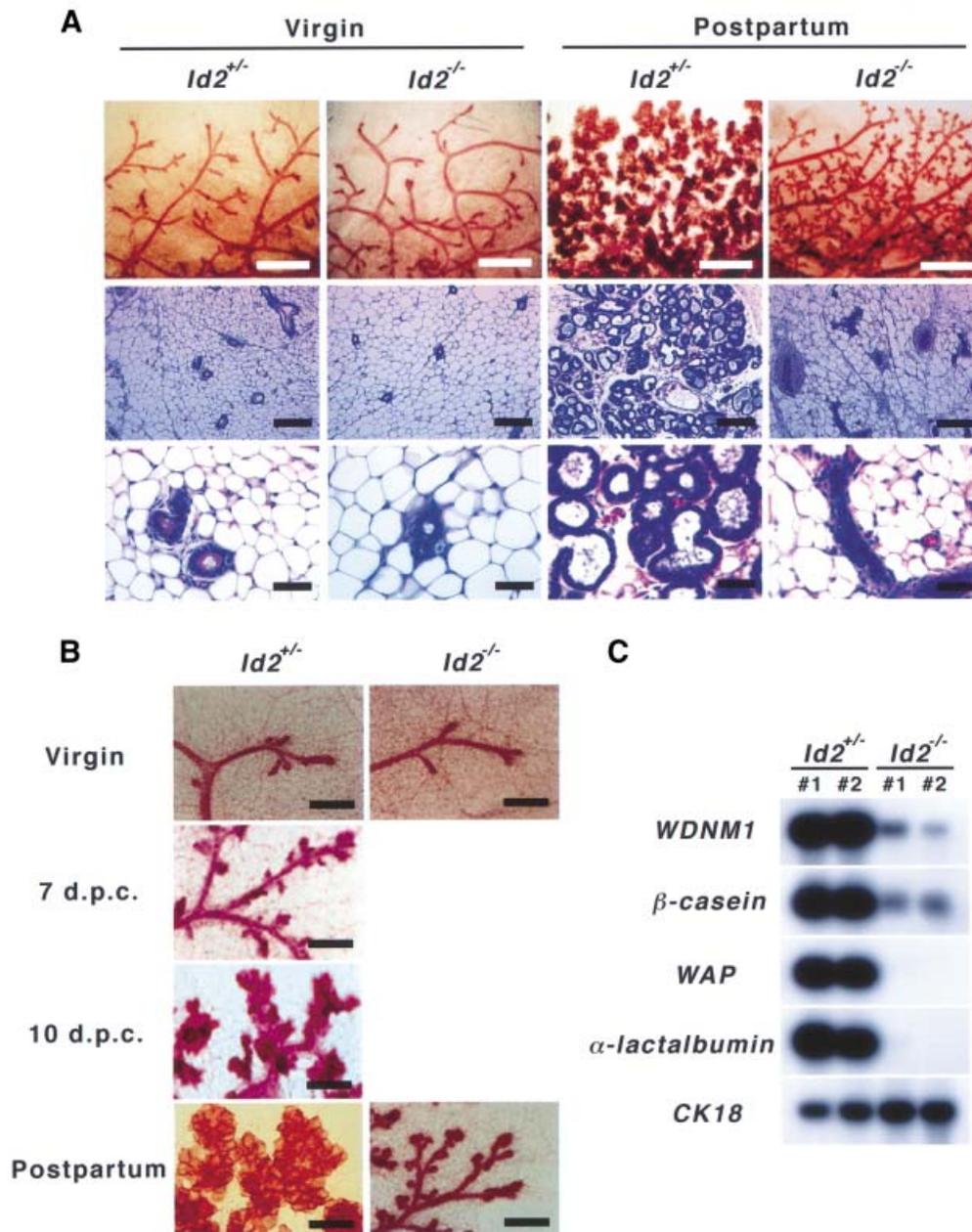
Maturation of mammary glands during pregnancy requires complex hormonal stimulation. Epithelial-mesenchymal interaction is important for mammary gland development not only during generation of the glands but also in pregnancy (Neville and Daniel, 1987). To determine which component is responsible for the lactation defect, we performed transplantation experiments (Figure 2). A cleared fat pad was prepared by completely removing the area containing endogenous mammary glands at 3 weeks of age. As shown in Figure 2A, small pieces of mammary glands were then implanted into a cleared fat pad and allowed to develop into secondary mammary glands in the host. Using this technique, the epithelium contained in the transplanted mammary gland can grow and penetrate the host fat pad and become associated with the stroma of the host

(Hoshino *et al.*, 1976; Robinson and Hennighausen, 1997), although a contribution of stromal cells of donor origin is not completely excluded. These mice with chimeric mammary glands were mated and mammary tissues were analyzed on the day of delivery. *Id2*<sup>+/-</sup> glands showed lobulo-alveolar development not only in heterozygous but also in homozygous recipients, similar to the glands of normal pregnant mice (Figure 2B). These results indicate that normal hormonal function is preserved in *Id2*<sup>-/-</sup> females and suggested that *Id2*<sup>-/-</sup> stroma seem to be able to interact normally with donor type epithelial cells. On the other hand, transplanted *Id2*<sup>-/-</sup> epithelia failed to develop lobulo-alveolar tissues in both heterozygous and homozygous mice (Figure 2B), suggesting that *Id2*<sup>-/-</sup> mammary epithelial cells cannot respond to the normal environment of the host in the presence of hormones and mammary stroma. Taken together, the transplantation experiments suggest that the basis of the lactation defect of *Id2* null mutants is intrinsic to the mammary epithelial cells.

To support this notion we analyzed the spatio-temporal expression pattern of *Id2*. *In situ* hybridization revealed expression of *Id2* in cells that are positive for *cytokeratin 18 (CK18)* (Figure 3A), indicating that *Id2* is expressed in ductal and glandular epithelia of mouse mammary glands. Moreover, northern blot analyses revealed that *Id2* expression is up-regulated during pregnancy and reaches its maximal level at ~10 d.p.c. (Figure 3B). The stage that *Id2* null mammary glands show an attenuation of gland development corresponds almost to the peak of *Id2* expression (Figure 1A and B). These results confirmed that mammary epithelial cells are responsible for the lactation defect.

*Id* proteins have been shown to inhibit cell differentiation in many cell culture systems (reviewed in Norton *et al.*, 1998), raising the possibility that the loss of *Id2* leads to premature differentiation of epithelial cells and exit from the cell cycle. Incomplete differentiation of *Id2*<sup>-/-</sup> mammary glands at parturition, as shown in Figure 1A and B, may be the result of precocious maturation of epithelial cells during the early phase of pregnancy. We performed *in situ* hybridization analyses of mammary glands at 7 d.p.c. with probes for milk protein genes such as *WDM1*,  $\beta$ -casein, *WAP* and  $\alpha$ -lactalbumin. *Id2*<sup>-/-</sup> glands expressed *WDM1* and  $\beta$ -casein transcripts but the expression levels were much lower than those in *Id2*<sup>+/-</sup> glands. Neither *WAP* nor  $\alpha$ -lactalbumin was detected in heterozygous and homozygous glands (Figure 4). These observations were compatible with northern blot analyses of mammary glands on the day of delivery, as shown in Figure 1C, and demonstrate no sign of precocious maturation in *Id2* null mammary cells. Similar results were obtained in specimens at 10 d.p.c. (data not shown). Thus, it is unlikely that the defective alveolar development in *Id2*<sup>-/-</sup> glands is due to premature differentiation of epithelial cells.

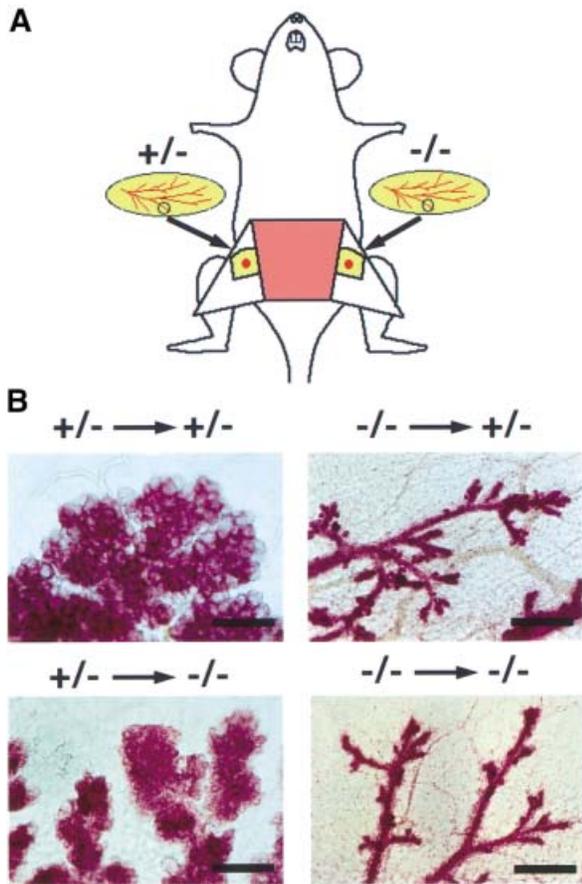
Next, bromodeoxyuridine (BrdU) incorporation and terminal deoxynucleotidyltransferase-mediated dUTP end-labeling (TUNEL) assays were performed to determine whether the decreased size of *Id2* null glands was the result of attenuated cell proliferation or an increased rate of cell elimination by apoptosis (Figure 5A). Several reports have indicated that *Id2* is a stimulator of the G<sub>1</sub>/S



**Fig. 1.** Impaired mammary gland development in *Id2*<sup>-/-</sup> mice during pregnancy. (A) (Upper) Whole mount analyses of mammary glands by carmine alum staining (lower magnification, scale bars represent 500  $\mu$ m). (Middle and lower) Histological analyses of mammary glands by hematoxylin and eosin staining. The middle and lower panels are lower and higher magnification, respectively (scale bars represent 400 and 100  $\mu$ m, respectively). Virgin glands of 8-week-old females (left) and glands on the day of delivery (right). Genotypes are indicated at the top of the panels. (B) Whole mount analyses of mammary glands (higher magnification). The glands of *Id2*<sup>+/+</sup> (left) (virgin, 7 and 10 d.p.c. and 1 day post-partum) and *Id2*<sup>-/-</sup> (right) (virgin and 1 day post-partum) mice are shown. Scale bars represent 50  $\mu$ m. (C) Expression of milk protein genes. Total RNA extracted from mammary glands on the day of delivery was analyzed by northern hybridization. *WDM1* and  $\beta$ -casein were used as early markers of milk genes. As markers for later stages,  $\alpha$ -lactalbumin and *WAP* were used. *CK18* is a marker for epithelial cells. Probes used are indicated on the left. Two examples are shown for each genotype group.

transition and able to enhance cell proliferation (Barone *et al.*, 1994; Hara *et al.*, 1994; Iavarone *et al.*, 1994; Lasorella *et al.*, 1996). The percentages of BrdU-positive epithelial cells in *Id2*<sup>+/+</sup> and *Id2*<sup>-/-</sup> mice were  $11.8 \pm 2.3$  and  $2.0 \pm 0.8\%$  at 7 d.p.c., respectively, demonstrating a marked contrast in the number of proliferating epithelial cells (Figure 5A). In contrast, no significant differences were detected at 4, 10 and 17 d.p.c. and more BrdU-positive epithelial cells were observed in *Id2*<sup>-/-</sup> mammary

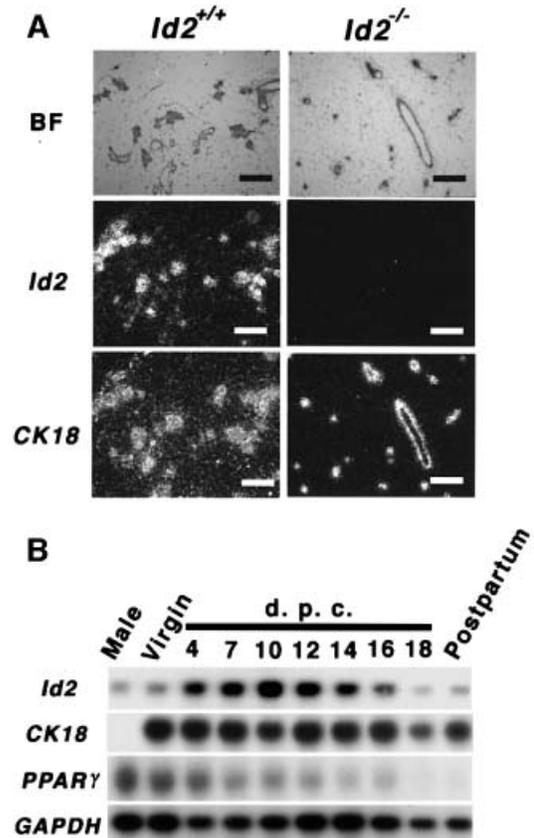
glands at 14 d.p.c. ( $4.0 \pm 1.1$  and  $7.6 \pm 0.4\%$  in *Id2*<sup>+/+</sup> and *Id2*<sup>-/-</sup> epithelial cells, respectively) (Figure 5A). On the other hand, the TUNEL assay detected no significant differences at 4, 7 and 10 d.p.c. between *Id2*<sup>+/+</sup> and *Id2*<sup>-/-</sup> mammary epithelia, but the percentage of apoptotic cells increased in *Id2*<sup>-/-</sup> mammary epithelia at 14 d.p.c., as shown in Figure 5A ( $0.5 \pm 0.04$  and  $2.5 \pm 0.2\%$  in *Id2*<sup>+/+</sup> and *Id2*<sup>-/-</sup> epithelial cells, respectively). This tendency was also observed at 17 d.p.c., although it is not statistically



**Fig. 2.** Intrinsic defect in *Id2*<sup>-/-</sup> mammary epithelial cells. (A) Schematic presentation of transplantations. Tissues prepared from *Id2*<sup>+/-</sup> and *Id2*<sup>-/-</sup> donors were transplanted into right and left cleared fat pads, respectively, of a recipient. (B) Whole mount analyses of engrafted mammary glands. Transplanted mammary glands were stained with carmine alum on the day of delivery. Directions of transplantation are indicated on the top of each panel. Scale bars represent 50 μm.

significant due to a variation among *Id2*<sup>-/-</sup> mammary epithelia. Thus, the defective mammapoiesis in the mutant mice seems to be derived from a combinatorial effect of a proliferation defect and apoptosis, which are observed in the early and late phases of pregnancy, respectively.

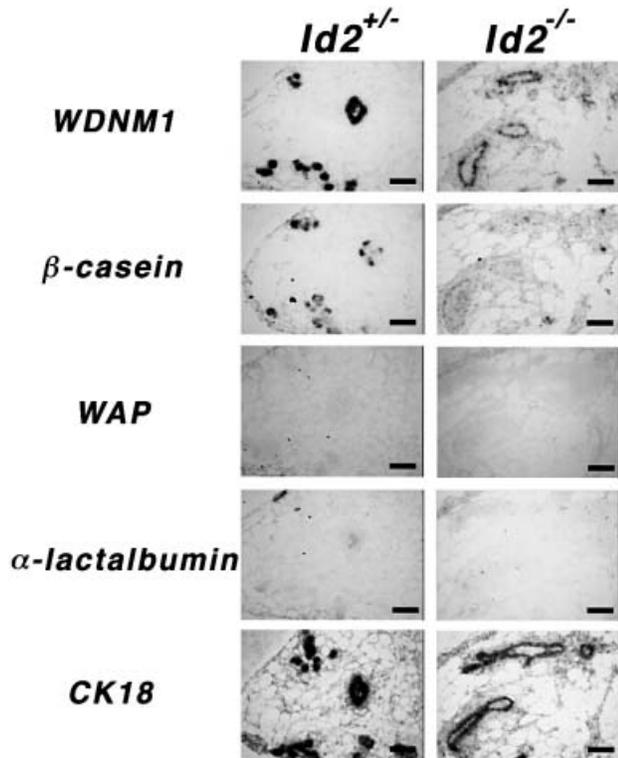
To investigate the molecular event underlying the proliferation defect in *Id2*<sup>-/-</sup> mammary epithelia in the early phase of pregnancy, expression levels of cell cycle regulators were next analyzed. Cyclin-dependent kinase inhibitors play important roles in cell cycle regulation (Sherr and Roberts, 1999) and Id proteins have been reported to be involved in the regulation of p16<sup>INK4a</sup> and p27<sup>Kip1</sup> protein expression (Lyden *et al.*, 1999) and p21<sup>WAF1</sup> mRNA transcription (Prabhu *et al.*, 1997). We therefore focused on their expression levels in mammary glands in early pregnancy. As shown in Figure 5B, p21<sup>WAF1</sup> mRNA in *Id2*<sup>-/-</sup> glands was expressed at 2- to 5-fold higher levels than that in *Id2*<sup>+/-</sup> glands. p27<sup>Kip1</sup> protein, which is regulated by translational controls (Hengst and Reed, 1996; Millard *et al.*, 1997) and post-translational mechanisms (Pagano *et al.*, 1995; Sheaff *et al.*, 1997), was up-regulated in *Id2* null mammary tissues, when compared with that of heterozygous glands



**Fig. 3.** *Id2* expression in mammary glands. (A) *In situ* hybridization analyses of mammary glands at 7 d.p.c. Serial sections were hybridized to a *CK18* antisense probe. (Left) Results for *Id2*<sup>+/-</sup> mammary glands (Right) Results for *Id2*<sup>-/-</sup> mammary glands. Probes used are indicated on the left. Scale bars represent 400 μm. BF, bright field. (B) Northern blot analysis of the time course of *Id2* expression in mammary glands. *CK18* and *PPARγ* were used as markers of epithelial cells and adipocytes, respectively. As a loading control for RNA in each lane, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used. Probes used are indicated on the left.

(3- to 6-fold, as demonstrated in Figure 5B). Expression of p16<sup>INK4a</sup> was not detected even by RT-PCR analysis (data not shown), indicating that its level is substantially low in pregnant mammary glands. Thus, the proliferation defect of *Id2* null mammary epithelial cells is accompanied by up-regulation of p21<sup>WAF1</sup> and p27<sup>Kip1</sup>.

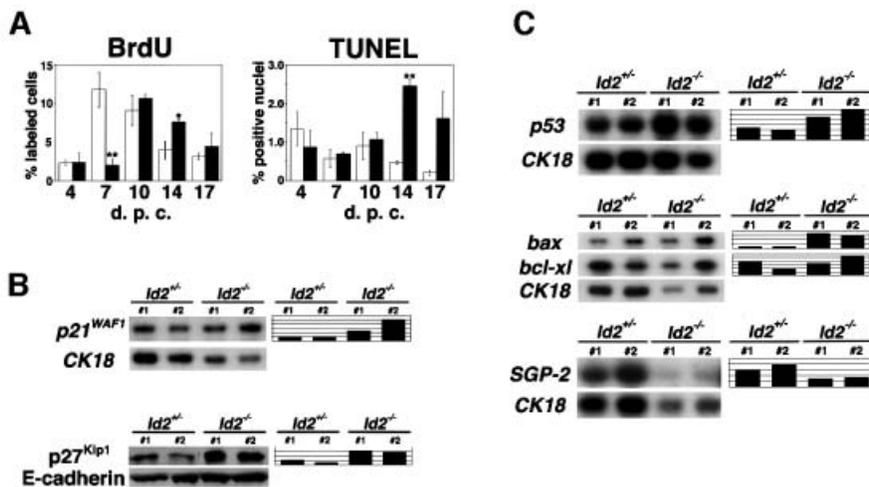
To explore the molecular basis of the enhanced apoptosis of *Id2* null mammary cells in the late phase of pregnancy, p53 expression in 14 d.p.c. mammary glands was first analyzed by northern analysis (Figure 5C). Its expression level in *Id2*<sup>-/-</sup> mammary glands was elevated 2- to 3-fold (Figure 5C), suggesting that the p53-dependent pathway is involved in the cell death of *Id2*<sup>-/-</sup> mammary epithelia. Examination of the expression level of *bax*, which is a pro-apoptotic gene belonging to the Bcl-2 family and transactivated by p53 (Oltvai *et al.*, 1993; Miyashita and Reed, 1995), revealed that there was a 7- to 8-fold higher level in *Id2*<sup>-/-</sup> mammary glands than in *Id2*<sup>+/-</sup> glands (Figure 5C). In contrast, no remarkable difference was observed in *bcl-xl* and *bcl-2* mRNA expression (Figure 5C and data not shown), anti-apoptotic members of the Bcl-2 family (Vaux *et al.*, 1988; Boise *et al.*, 1993; Gonzalez-Garcia *et al.*, 1994). *bcl-xs* mRNA was not



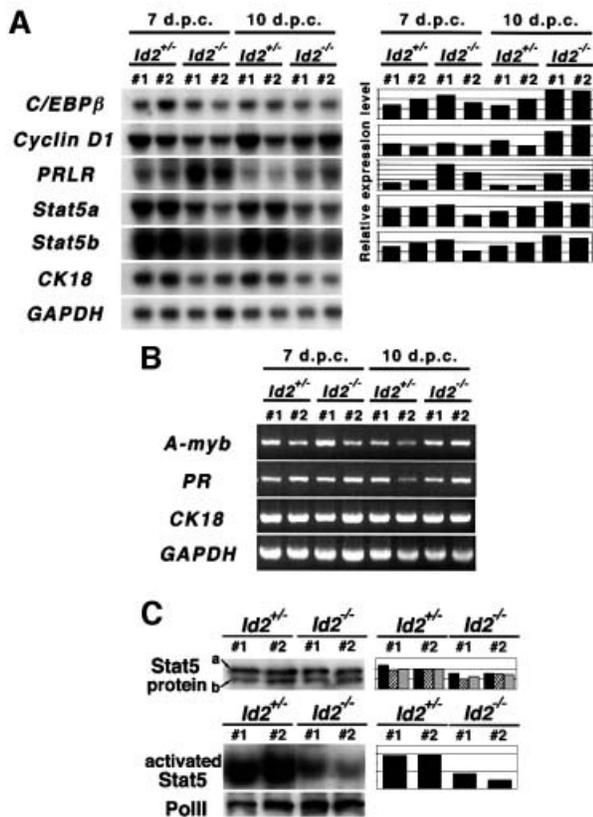
**Fig. 4.** No precocious maturation in *Id2*<sup>-/-</sup> mammary glands. Mammary glands of *Id2*<sup>+/+</sup> (left) and *Id2*<sup>-/-</sup> (right) mice at 7 d.p.c. were analyzed by *in situ* hybridization. Probes used were the genes encoding milk proteins, *WDNM1*, *β-casein*, *WAP* and *α-lactalbumin*, as indicated on the left. *CK18* was used as a marker of epithelial cells. Scale bars represent 200 μm.

detected, even after extended exposure (data not shown). These observations suggested that *Id2* plays a role in late pregnant mammary cells as a cell survival factor by inhibiting the p53–Bax pathway. Elevated expression of *p53* and *bax* is reminiscent of the situation that occurs during involution. Involution is a step by which secretory mammary epithelia are removed by massive apoptosis and lactation is terminated (Neville and Daniel, 1987). During involution the gene encoding sulfated glycoprotein-2 (*SGP-2*) (Buttayan *et al.*, 1989) is strongly induced, in addition to *p53* and *bax* (Strange *et al.*, 1992; Heermeier *et al.*, 1996; Metcalfe *et al.*, 1999). As shown in Figure 5C, however, *SGP-2* was not induced in *Id2*<sup>-/-</sup> mammary glands, excluding the possibility of an involution-like mechanism.

Recent studies have revealed genes that are essential for mammary gland development during pregnancy. Examples include *cyclin D1*, *A-myb*, *progesterone receptor*, *prolactin receptor*, *Stat5a*, *Stat5b* and *C/EBPβ* (Fantl *et al.*, 1995, 1999; Lydon *et al.*, 1995; Robinson *et al.*, 1995; Sicinski *et al.*, 1995; Humphreys *et al.*, 1997; Liu *et al.*, 1997; Ormandy *et al.*, 1997; Toscani *et al.*, 1997; Briskin *et al.*, 1998, 1999; X.Liu *et al.*, 1998; Seagroves *et al.*, 1998; Teglund *et al.*, 1998). To examine the relationship of *Id2* to these genes, northern blot and RT-PCR analyses were performed in mammary glands at 7 and 10 d.p.c. (Figure 6). Northern blotting indicated no reduction in gene expression of *C/EBPβ*, *cyclin D1*, *prolactin receptor*, *Stat5a* or *Stat5b* at 7 and 10 d.p.c. Rather, up-regulation was observed for *C/EBPβ* and *cyclin D1* at 10 d.p.c. (>2-fold compared with the control).



**Fig. 5.** Defective proliferation and enhanced apoptosis of pregnant *Id2*<sup>-/-</sup> mammary epithelial cells. (A) BrdU labeling index (left) and TUNEL assay (right). In these analyses >4000 nuclei of epithelial cells were examined in each of the samples taken at different days of pregnancy as indicated, and the percentages of BrdU-labeled or TUNEL-positive cells were determined. Columns represent the mean percentages of *Id2*<sup>+/+</sup> (white) and *Id2*<sup>-/-</sup> (black) mammary epithelia. Standard errors of the mean (*n* = 3) are shown. Student's *t*-test was used for statistical analysis. Single and double asterisks indicate *P* values of <0.03 and <0.01, respectively. (B) Up-regulation of p21<sup>WAF1</sup> and p27<sup>Kip1</sup> in *Id2*<sup>-/-</sup> mammary glands. Total RNA or protein was extracted from mammary glands at 7 d.p.c. (Upper) RNase protection assays for p21<sup>WAF1</sup> and *CK18* in *Id2*<sup>+/+</sup> and *Id2*<sup>-/-</sup> mammary glands. Relative expression levels of p21<sup>WAF1</sup> to *CK18* are indicated on the right. (Lower) Western blot analysis of p27<sup>Kip1</sup> and E-cadherin. Relative expression levels of p27<sup>Kip1</sup> to E-cadherin are indicated on the right. The relative expression level of heterozygous mouse no. 2 is designated 100%. Genotypes are indicated at the top of each panel. (C) Examination of expression levels of several apoptosis-related genes in *Id2* null mammary glands. Total RNA was prepared from 14 d.p.c. mammary glands. (Upper) *p53* and *CK18* expression assays determined by northern analysis. (Middle) RNase protection assays of *bax*, *bcl-x1* and *CK18*. (Lower) Northern blot analysis of *SGP-2* and *CK18*. Relative expression levels of *p53*, *bax* and *bcl-x1* compared with *CK18* are shown on the right of each panel. The expression level of heterozygous mouse no. 2 is designated 100%. Genotypes are indicated at the top of each panel.



**Fig. 6.** *Id2* is a downstream or parallel factor of genes indispensable for lobulo-alveolar expansion. Expression of genes and proteins, indicated on the left, was analyzed in *Id2*<sup>+/+</sup> and *Id2*<sup>-/-</sup> mammary glands at the indicated d.p.c. (A) Northern analyses for *C/EBPβ*, prolactin receptor (*PRLR*), *Stat5a* and *Stat5b* expression. *CK18* is an epithelial cell marker and *GAPDH* was used to quantitate total amount of RNA. The expression levels of *C/EBPβ*, *PRLR*, *Stat5a* and *Stat5b* relative to *CK18* expression are shown on the right. The expression level of each gene in the 10 d.p.c. mammary glands of heterozygote no. 2 is designated 100%. (B) RT-PCR analyses for *A-myb* and progesterone receptor (*PR*) expression. *CK18* was used as an epithelial cell marker. As an internal control, *GAPDH* was included. (C) Down-regulation of the DNA binding activity of Stat5. (Upper) Protein levels of Stat5 in whole cell extracts of 7 d.p.c. mammary glands were analyzed by immunoblotting. The samples used in Figure 5B were subjected to this analysis. Positions of proteins Stat5a and Stat5b are indicated by a and b, respectively. Relative expression levels of Stat5a (black columns), Stat5b (hatched columns) and the sum of Stat5a and Stat5b (dotted columns) to E-cadherin are indicated on the right. (Lower) The DNA binding activity of Stat5 in nuclear extracts of 7 d.p.c. mammary glands was analyzed by EMSA with a Stat5 recognition sequence. The protein levels of RNA polymerase II, analyzed by western blotting, serves as a loading control of nuclear extract. Quantitated and normalized DNA binding activity is shown on the right. In all panels two examples are shown for each genotype group.

Expression of *prolactin receptor* at 7 and 10 d.p.c. was also higher than that of the control, 2- and 4-fold, respectively (Figure 6A). Additionally, RT-PCR analyses demonstrated similar levels of expression of the *A-myb* and *progesterone receptor* genes (Figure 6B). These results revealed that *Id2* is not an upstream factor of these genes in terms of transcriptional regulation.

Among the molecules analyzed for their gene expression, Stat5 is an intracellular transducer of the signal induced by prolactin (Wakao *et al.*, 1994) and its activity as a transcription factor depends on phosphorylation of tyrosine residues (Hennighausen *et al.*, 1997). We there-

fore tried to determine the actual activity of Stat5 (Figure 6C). For this the electromobility shift assay (EMSA) was applied, because preliminary experiments indicated that EMSA is more sensitive than detection of phosphotyrosine residues of activated Stat5 by immunoprecipitation-western blot analysis (data not shown). The amounts of Stat5a and Stat5b proteins in whole cell extracts were slightly decreased in *Id2*<sup>-/-</sup> mammary glands (Figure 6C, upper). The total DNA-binding activity of Stat5 in nuclear extracts of *Id2*<sup>-/-</sup> mammary glands, however, exhibited a further decrease to ~40% of that of the control (Figure 6C, lower). These results suggest that decreased activity of Stat5, at least in part, participates in the lactation defect in *Id2*-deficient mice. Moreover, this raises the interesting possibility that *Id2* plays a role in the Stat5 signaling pathway.

## Discussion

We have shown that *Id2*-deficient female mice exhibit a severe lactation defect. Transplantation experiments suggest that the defect is intrinsic to mammary epithelial cells. This was confirmed by expression analyses of *Id2* during pregnancy. Impaired cell proliferation and enhanced apoptosis of *Id2*-deficient mammary epithelia are evident in the early and late phases of pregnancy, respectively. *Id2*-deficient mammary epithelia remain poorly differentiated. Analyses of the expression levels of genes required for mammary gland development suggest that *Id2* possibly acts downstream or parallel to these factors in mammary epithelia during pregnancy. In addition, the functional activity of Stat5 is decreased in *Id2*<sup>-/-</sup> mammary glands, implying the possible participation of *Id2* in the Stat5 signaling pathway. Our results demonstrate that *Id2* is involved in cell cycle progression and cell survival in pregnant mammary glands, enabling the expansion of epithelial cells during pregnancy.

*Id* proteins have been proposed to possess two functions; inhibition of cell differentiation and stimulation of cell proliferation (reviewed in Norton *et al.*, 1998), both of which are mutually related. The former activity is executed by suppression of bHLH factors through interaction with the HLH regions, which has been considered to be a major role of *Id* proteins, particularly during development (Benezra *et al.*, 1990; Norton *et al.*, 1998). For the latter function an antagonistic activity of *Id2* against the retinoblastoma protein and release of the suppression of p21<sup>WAF1</sup> transcription by bHLH factors have been reported (Sorrentino *et al.*, 1990; Iavarone *et al.*, 1994; Prabhu *et al.*, 1997), although the detailed mechanisms are still largely unclear. The former function allows us to speculate that a deficiency of *Id2* would lead to accelerated or premature differentiation of mammary glands. In fact, precocious maturation of neuroepithelia is observed in *Id1*<sup>-/-</sup>*Id3*<sup>-/-</sup> mice, in association with up-regulation of expression of neuronal bHLH factors and other markers of neuronal differentiation (Lyden *et al.*, 1999). Conversely, an overexpression study of *Id1* in a mammary gland-derived cell line shows disturbed differentiation (Desprez *et al.*, 1995). In this context we speculated that premature differentiation might be a cause of the lactation defect in *Id2* mutant mice and thus that a bHLH factor(s) could participate in mammary gland

development during pregnancy. However, no signs of precocious maturation were observed by *in situ* analyses of genes encoding milk proteins. We have also failed so far to identify any tissue-specific bHLH transcription factors that may be potentially involved in mammary gland development in pregnancy (S.Mori and Y.Yokota, unpublished data). This would suggest that the function of Id2 in pregnant mammary glands appears to be to regulate cell differentiation by controlling the activity of a potential bHLH factor, which in turn promotes differentiation of mammary glands. However, we cannot exclude the possibility of the involvement of a bHLH factor that suppresses differentiation of mammary glands. By inhibiting the activity of such a bHLH factor, Id2 may promote cell differentiation of mammary glands during normal mammary gland development.

BrdU incorporation experiments indicated that *Id2* null mammary glands exhibit a defect in cell proliferation at 7 d.p.c., during the early phase of pregnancy. This time point corresponds to the induction of *Id2* expression in wild-type mammary glands as shown by northern analysis. *Id2*<sup>-/-</sup> mammary glands remained attenuated at this stage, as demonstrated morphologically by whole mount staining and biochemically by the expression of milk protein genes. Proliferation of mammary epithelial cells during pregnancy reaches its maximum in the early phase, coinciding with high concentrations of serum hormones such as prolactin and progesterone (Virgo and Bellward, 1974; Talamantes *et al.*, 1984; Neville and Daniel, 1987). Thus, the defect identified in *Id2* null mutant mice suggests that the function of Id2 in the early phase of pregnancy is to stimulate cell cycle progression of mammary epithelial cells.

The high levels of p21<sup>WAF1</sup> and p27<sup>Kip1</sup> expression are compatible with the observed cell cycle arrest in *Id2* null mammary glands. These observations suggest that activation of cyclin-dependent kinase inhibitors is responsible for the attenuated cell proliferation in *Id2* null mammary epithelia during early pregnancy. Overexpression of the *E2A* gene in cultured cells enhances p21<sup>WAF1</sup> transcription resulting in cell cycle arrest, which can be prevented by Id1 (Peverali *et al.*, 1994; Prabhu *et al.*, 1997). A similar effect is also induced by MyoD (Sorrentino *et al.*, 1990). As the activity of bHLH factors such as the *E2A* gene products and MyoD is negatively regulated by Id proteins (Benezra *et al.*, 1990), it is natural to think that loss of Id2 results in functional up-regulation of bHLH factors and therefore up-regulation of p21<sup>WAF1</sup> transcription. However, although *E2A* and other E protein genes are actually expressed in mammary glands during pregnancy at constant levels (S.Mori and Y.Yokota, unpublished observation), a tissue-specific bHLH factor is less likely to be present in mammary glands, as discussed above. Homo- or heterodimers consisting of E proteins, which are usually formed in lymphoid cells (Bain *et al.*, 1993; Benezra, 1994; Shen and Kadesch, 1995), may be involved in activation of p21<sup>WAF1</sup> transcription. Alternatively, further investigations may identify a bHLH factor in pregnant mammary glands. p21<sup>WAF1</sup> transcription is also known to be activated by p53 (el-Deiry *et al.*, 1993) and Stat5 (Matsumura *et al.*, 1997). In *Id2* null mammary glands at 7 d.p.c. p53 expression is not elevated (S.Mori and Y.Yokota, unpublished observation) and the DNA-binding

activity of Stat5 is decreased, making it unlikely that these factors are involved. The protein level of p27<sup>Kip1</sup>, on the other hand, is regulated by several mechanisms, such as degradation by the ubiquitin-proteasome pathway through phosphorylation of p27<sup>Kip1</sup> by cyclin E-cyclin-dependent kinase 2 complexes (Pagano *et al.*, 1995; Sheaff *et al.*, 1997). The fact that developing neuroepithelia of *Id1/Id3* compound null mutant embryos show a similar enhancement of p27<sup>Kip1</sup> (Lyden *et al.*, 1999) implies a common mechanism in altering the p27<sup>Kip1</sup> level by Id proteins, although the detailed mechanism underlying the up-regulation of p27<sup>Kip1</sup> is unclear at present.

In the late phase of pregnancy *Id2*<sup>-/-</sup> mammary epithelia exhibit significant BrdU incorporation and enhanced apoptosis. These suggest that the absence of Id2 does not affect the proliferation of mammary cells but that Id2 additionally plays a role as a survival factor *in vivo*, at least for late pregnant mammary epithelia. Considering that augmented apoptosis is observed on overexpression of Id proteins (Florino *et al.*, 1998; Norton and Atherton, 1998), an appropriate level of Id2 protein may be important for cell growth and survival. Interestingly, cyclin D1, which is essential for development of mammary glands during pregnancy (Sicinski *et al.*, 1995; Fantl *et al.*, 1999), has been reported to be required for the viability of retinal cells (Ma *et al.*, 1998). These observations imply that Id2 and cyclin D1 share similar functions in the regulation of cell proliferation and cell death. We have so far failed, however, to detect a physical interaction between Id2 and cyclin D1 using the yeast two-hybrid system (M.Tanji and Y.Yokota, unpublished observation).

In 7 d.p.c. *Id2* null mammary glands, expression of the early maturation marker genes *WDM1* and  $\beta$ -casein is hardly detectable and apoptosis of epithelial cells is not significant. These data do not support the notion that differentiated *Id2*<sup>-/-</sup> mammary epithelia are removed by apoptosis in the early phase of pregnancy. In the late phase of pregnancy, on the other hand, *WAP* and  $\alpha$ -lactalbumin expression commence in the glandular structure (Robinson *et al.*, 1995), which fails to develop in *Id2*-deficient mammary glands. The loss of the alveolar component seems to be responsible for the impaired expression of these late milk proteins. Alternatively, differentiated cells expressing *WAP* and  $\alpha$ -lactalbumin may be eliminated by apoptosis in *Id2* null mammary glands.

Northern blot and RT-PCR analyses demonstrated that expression of genes involved in lobulo-alveolar development was not significantly reduced in *Id2*<sup>-/-</sup> mammary glands and, instead, genes such as *prolactin receptor* were up-regulated, indicating that expression of these genes is not dependent on the activity of Id2. It is tempting to think that signals elicited by hormonal stimuli converge on Id2 to promote cell cycle progression of mammary epithelial cells during early pregnancy. Alternatively, Id2 may act in parallel with molecules involved in lobulo-alveolar development of pregnant mammary glands. Among the genes analyzed, *prolactin receptor* was observed as being up-regulated at both 7 and 10 d.p.c. in *Id2*<sup>-/-</sup> mammary glands. This may reflect the immature state of *Id2* null mammary glands, since *prolactin receptor* mRNA expression is gradually down-regulated as pregnancy proceeds in wild-type mice (S.Mori and Y.Yokota, unpublished observation).

In fact, the DNA-binding activity of Stat5 is decreased in *Id2* null mammary glands, as demonstrated by EMSA, despite the increased *prolactin receptor* expression revealed by northern analyses and the normal hormonal environment as demonstrated by transplantation experiments. The mammary phenotype of *Stat5a*<sup>-/-</sup> mice (Liu *et al.*, 1997; X.Liu *et al.*, 1998; Teglund *et al.*, 1998) is less severe than that of *Id2*<sup>-/-</sup> mice, suggesting that decreased activity of Stat5 is not a major cause of the lactation defect of *Id2*-deficient mice. Since we have failed so far to detect a direct association between *Id2* and Stat5 by mammalian two-hybrid assays (S.Mori and Y.Yokota, unpublished observation), an indirect interaction might be involved in Stat5 signaling via molecules such as SOCS/CIS (Yoshimura *et al.*, 1995; Starr *et al.*, 1997) and PIAS (Chung *et al.*, 1997; B.Liu *et al.*, 1998), which regulate Stat5 activity at several steps (Starr and Hilton, 1999).

Among the genes required for lobulo-alveolar development, of note is *cyclin D1*, a cell cycle regulator, as discussed above. In ~30–50% of breast cancer cases *cyclin D1* is overexpressed and ~15–20% of cases demonstrate amplification of the *cyclin D1* gene locus (Lammie *et al.*, 1991; Bartkova *et al.*, 1994; Gillett *et al.*, 1994; reviewed in Barnes and Gillett, 1998). In addition, transgenic mice expressing the *cyclin D1* gene under control of the MMTV LTR develop mammary adenoma and hyperplasia (Wang *et al.*, 1994). *Id2* has been shown to be overexpressed in pancreatic cancers (Kleeff *et al.*, 1998; Maruyama *et al.*, 1999). *Id2*, which has the ability to stimulate cell cycle progression, may also be involved in the development of breast cancer. Preliminary overexpression experiments of *Id2* using HC11, a mammary epithelial cell line, have failed so far to show anchorage-independent growth or tumorigenic potential when injected into nude mice (S.Mori and Y.Yokota, unpublished observation), as in the case of *cyclin D1* (Han *et al.*, 1995, 1996). We are now examining a role of *Id2* in human breast cancer and establishing transgenic mouse lines that target *Id2* expression in the mammary gland. These investigations would clarify a potential role of *Id2* in mammary carcinogenesis, combined with studies using crosses of *Id2*<sup>-/-</sup> mice with mouse models for breast cancer, such as MMTV–*cyclin D1* mice.

## Materials and methods

### Mice

*Id2*-deficient mice of mixed genetic background (129/Sv × NMRI) were used in most of the experiments (Yokota *et al.*, 1999). For transplantation experiments mice with the 129/Sv background were used. No strain-dependent variation has been identified in mammary glands.

### Tissue preparations and histology

Inguinal mammary glands no. 4 were used for all experiments. For histological analyses mice were anesthetized with diethyl ether and perfused with phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA). Mammary glands were dissected out and post-fixed with PBS containing 4% PFA and 0.2% Tween-20 on ice overnight. They were embedded in paraffin according to standard procedures. Sections of 8 μm were used for each assay. Whole mount staining of mammary glands was performed as previously described (Horseman *et al.*, 1997).

### Transplantation experiments

Transplantations were carried out as described (DeOme *et al.*, 1959; Hoshino *et al.*, 1976). Briefly, fat pads containing inguinal mammary glands no. 4 on both sides were removed from 3-week-old female

recipient mice. Complete removal of the mammary glands was confirmed by whole mount staining of dissected mammary glands no. 4. Two or three pieces of mammary tissue isolated from inguinal glands (approximately <2 mm in diameter) of 6-week-old mice were inserted into the cleared fat pads of recipient mice. Tissues prepared from *Id2*<sup>+/-</sup> and *Id2*<sup>-/-</sup> donors were transplanted into the right and left cleared fat pads, respectively, of the recipient. The transplanted mammary tissue formed chimeric secondary mammary glands mainly consisting of a donor-derived epithelial component and host-derived stroma (Hoshino *et al.*, 1976; Robinson and Hennighausen, 1997). However, a minor contribution of donor-derived stroma and fatty tissues is not completely excluded. Six weeks after the operation, the recipients were mated and the grafted fat pads were harvested on the day of delivery for whole mount staining.

### Cell proliferation and TUNEL assays

*In vivo* incorporation of BrdU was used for cell proliferation assays. Two hours before killing, BrdU (120 μg/g body wt) was injected i.p. into mice on the indicated days of pregnancy. After embedding the mammary glands in paraffin and sectioning, incorporated BrdU was detected with anti-BrdU antibody conjugated to horseradish peroxidase (Boehringer Mannheim) according to the manufacturer's instructions.

Apoptotic cells were determined by the TUNEL method with an *In Situ* Cell Death Detection Kit (Boehringer Mannheim), combined with their apoptotic morphology.

Samples were counterstained with hematoxylin. Serially sectioned samples were examined by these analyses.

### Northern blot, RNase protection and RT-PCR analyses

The northern blot and RNase protection assays were carried out using standard protocols as previously described (Chanda, 1995; Narumi *et al.*, 2000). Probes for northern analyses were labeled with [<sup>32</sup>P]dCTP by the random priming method and those for RNase protection assays were prepared by *in vitro* transcription with [<sup>32</sup>P]UTP (Chanda, 1995). The probes used in this study were derived from the following plasmids: *Id2*, pRcCMV-*Id2*; *PPARγ*, pCMXmPPARγ; *cytokeratin 18*, pBluecBSE; *cyclin D1*, pcBZ05.4; *C/EBPβ*, pMSV/EBPβ; *Stat5a*, pME18S-*Stat5a*; *Stat5b*, pME18S-*Stat5b*; *PRLR*, pBS-mPRLR; p21<sup>WAF1</sup>, pBSmp21; *p53*, pp53-176; *bax*, pSFFVneo-HA-mBAX; *bcl-xL*, pSFFVneo-mBclXL; *bcl-2*, pSKMBBR; *SGP-2*, p1321. Plasmids carrying cDNAs for *WDM1*, *β-casein*, *α-lactalbumin*, *WAP* and *GAPDH* were obtained by RT-PCR using primers designed according to the reported sequences in DDBJ/EMBL/GenBank. The expression levels of various molecular markers were quantitated with a BAS5000 Image Analyzer (Fuji Film).

RT-PCR was carried out using a standard protocol. Primers were designed according to the reported sequences in DDBJ/EMBL/GenBank.

### Western blot and EMSA

Western blot analyses and EMSA were performed essentially as described previously (Narumi *et al.*, 2000). Whole cell lysate prepared using NP-40 lysis buffer (Nakayama *et al.*, 1996) was used for analyses of p27, E-cadherin and *Stat5a/5b* expression. E-cadherin was used as a marker of luminal epithelial cells of mammary glands (Radice *et al.*, 1997) and an internal control for whole cell lysates. RNA polymerase II was used as an internal control for nuclear extracts. The probe used in EMSA for detection of activated Stat5 was derived from the bovine *β-casein* promoter/enhancer region (Wakao *et al.*, 1994). The specificity of shifted bands was confirmed by a supershift assay with anti-*Stat5a/5b* specific antibodies (data not shown). Antibodies used in this study were anti-p27 rabbit antiserum (15606E; Pharmingen), anti-E-cadherin rat monoclonal antibody (ECCD2; TaKaRa), anti-*Stat5a* rabbit antiserum (L-20; Santa Cruz Biotechnology), anti-*Stat5b* mouse monoclonal antibody (G-2; Santa Cruz Biotechnology) and anti-RNA polymerase II rabbit antiserum (C-21; Santa Cruz Biotechnology). Enhanced chemiluminescence reagents (Dupont-NEN Life Science) were used for visualization. Quantitation for EMSA and western analyses was done with a BAS5000 system (Fuji Film) and a Fluor-S MultiImager (Bio-Rad), respectively.

### In situ hybridization

*In situ* hybridization was conducted as described (Mori *et al.*, 1999). In the case of digoxigenin (DIG)-labeled RNA probes, alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim) was used for detection. To enhance sensitivity, polyvinylalcohol was added to the color reaction (Yokota *et al.*, 1999). The sense probe of *CK18* was used as a negative control (data not shown).

## Acknowledgements

We thank T.Neuman, K.Umesono, M.Nozaki, H.Matsushime, S.L.Mcknight, A.Miyajima, M.Sasaki, O.Niwa, I.Matsumura, G.Nunez, Y.Tsujimoto and R.Strange for providing us with materials. We thank P. Gruss for support during the initial stages of this study, including generation of mutant mice. This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan (07CE2005, 06277102, 10670119 and 06NP1101). S.M. is supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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Received January 11, 2000; revised August 2, 2000;  
accepted September 15, 2000