Forced expression of cyclin D1 does not compensate for Id2 deficiency in the mammary gland

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Abstract Id2 and cyclin D1 share several biological activities, including inhibition of differentiation, stimulation of the G1–S transition in the cell cycle and stimulation of tumorigenesis. Mammary glands of Id2$^{-/-}$ mice display severely impaired lobulo-alveolar development during pregnancy, similarly to those of cyclin D1 null females. We investigated the functional relationship between Id2 and cyclin D1 in the mammary gland. Id2$^{-/-}$ mammary glands expressed a normal level of cyclin D1. No direct interaction of Id2 with cyclin D1 or its binding partner cdk4 was detected in mammalian two-hybrid assays. Ectopic expression of a cyclin D1 transgene did not rescue the mammary phenotype of Id2$^{-/-}$ mice. These results suggest that Id2 acts downstream or independently of cyclin D1 in the control of mammary cell proliferation during pregnancy.

Key words: Id2; Cyclin D1; Mammary gland

1. Introduction

Mammary epithelial cells proliferate rapidly during pregnancy under the influence of various hormones and growth factors and form an expanded glandular structure via the process of lobulo-alveolar development, providing a good experimental model system for studying the mechanism of cell proliferation in vivo [1]. Loss-of-function experiments in mice have revealed several genes that are involved in the process of lobulo-alveolar development and carcinogenesis [2–6].

The four Id proteins (Id1–Id4) are negative regulators of basic helix-loop-helix transcription factors that play a pivotal role in multiple processes of cell differentiation, proliferation and other functions [7,8]. They are crucial for cell proliferation due to their ability to stimulate the G1–S transition in the cell cycle [8–13], although the precise mechanism of this stimulation has not been fully elucidated. The in vivo functions of Ids in cell cycle control have been confirmed by gene-targeting experiments in mice [5,14,15]. We previously reported that Id2-deficient female mice show a lactation defect due to impaired lobulo-alveolar development of their mammary glands during pregnancy. The observed defect is intrinsic to epithelial cells and mostly due to the impaired proliferation of these cells during early pregnancy [5]. Interestingly, the impaired proliferation of Id2$^{-/-}$ mammary epithelial cells at 7 days post coitus (d.p.c.) is restored at 10 d.p.c. [5]. This time point coincides with the augmented expression of cyclin D1 in Id2$^{-/-}$ mammary epithelial cells [5], suggesting the possibility that forced expression of cyclin D1 can rescue the mammary defect of Id2$^{-/-}$ mice.

Cyclin D1 belongs to the D-type cyclins, and specifically heterodimerizes with and activates cyclin-dependent kinases (cdks), cdk4 or cdk6, in the G1 phase of mammalian cells [16–18]. Several lines of evidence have suggested functional similarity of cyclin D1 and Id2 in the cell cycle, although their relationship remains unclear. Of particular note, mice with cyclin D1 deficiency show a mammary phenotype similar to that of Id2 null mice [4,5]. This observation raises the possibility of the collaboration of Id2 with cyclin D1 in mammary gland development. On the other hand, it is reported that enhanced expression of Id2 is associated with an inhibition of cyclin D1 expression, raising another possibility that the balance between Id2 and cyclin D1 is important in cell cycle regulation [19].

In this study we investigated the functional relationship of Id2 and cyclin D1 in vivo using a genetic approach and found that crossing with mammary epithelial specific cyclin D1 transgenic mice does not rescue the phenotype of Id2-deficient mice. Our results suggest that Id2 functions independently or downstream of cyclin D1 in the control of proliferation of pregnant mammary epithelial cells in vivo.

2. Materials and methods

2.1. Mice

Mouse mammary tumor virus (MMTV)-D1-transgenic (FVB/N) and Id2-deficient mice (129/Sv×NMRI) have been described previously [6,20]. Transheterozygous females generated by crossing between the two mouse lines were further crossed with Id2$^{-/-}$ males to obtain mice heterozygous or nullizygous for Id2 and transgenic (+/Tg) or non-transgenic (+/+) for cyclin D1. No difference has been observed in mammary gland development between Id2$^{+/-}$ and Id2$^{+/+}$ female mice.

2.2. Tissue preparations and histology

Inguinal mammary glands (#4) were used for all experiments. Mammary glands were fixed with Carnoy's fixative for whole-mount analyses or with 4% paraformaldehyde in phosphate-buffered saline

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Abbreviations: AD, transactivation domain; BD, DNA binding domain; BrdU, bromodeoxyuridine; cdk, cyclin-dependent kinase; CK18, cytokeratin 18; d.p.c., days post coitus; MMTV, mouse mammary tumor virus; pRb, retinoblastoma protein; RT-PCR, reverse transcription-polymerase chain reaction; Tg, transgenic

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for bromodeoxyuridine (BrdU) incorporation analyses, and further processed as described previously [5]. For immunohistochemistry, 5-μm-thick paraffin-embedded sections of 7 d.p.c. mammary glands were reacted with antibodies against cyclin D1 (DCS-6, BD Biosciences Pharmingen) and cyclin A2 (C-19; Santa Cruz Biotechnology), followed by HRP-conjugated anti-rabbit IgG and anti-mouse IgG antibodies (EnVision, Dako), respectively. Detection was done as described [5].

2.3. RNase protection, reverse transcription-polymerase chain reaction (RT-PCR), Northern and Western blot analyses

RT-PCR, Northern blot and RNase protection assays were carried out using standard protocols as previously described [5,21]. The primer sets used in RT-PCR were designed according to the sequences deposited in GenBank. Cytokeratin 18 (CK18) cDNA, used in Northern blot analysis, was derived from pBluecBSE [22]. The template sets for RNase protection were mCYC-1 and mCYC-2 (BD Biosciences). Immunoblot analyses were performed as previously described [21]. Antibodies used in this study were FITC-labeled anti-cyclin D1 antibody (CC12F; Oncogene), anti-cyclin A2 antibody (C-19; Santa Cruz Biotechnology) and anti-E-cadherin antibody (ECCD-2; TaKaRa).

2.4. Mammalian two-hybrid assay

Plasmids were constructed with pcBZ05.4 (cyclin D1, [18]), pBS-cdk4 [18], pCMV-Id2 and pCMV-E12 [21] based on pCMV-AD and pCMV-BD (Stratagene). Plasmids were transfected into NIH3T3 cells by lipofection and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) as previously described [21].

3. Results

To elucidate the effect of Id2 deficiency on cell cycle regulation in pregnant mammary glands, we studied the expression of cyclins in virgin and 7 d.p.c. mammary glands of Id2-deficient mice, because mammary epithelial cell proliferation is stimulated in the early phase of pregnancy [1], when $Id2^{-/-}$ mice show a defect in mammary glands [5]. Pregnancy up-regulated the mRNA expression of cyclins A2, B1 and D1 more than two-fold in the control glands (Fig. 1A). The levels of cyclin mRNAs were not down-regulated in either virgin or 7 d.p.c. mammary glands of $Id2^{-/-}$ females (Fig. 1A), even though $Id2^{-/-}$ mammary epithelial cells clearly show retarded cell proliferation at 7 d.p.c. [5]. The protein level of cyclin D1 was also normal in $Id2^{-/-}$ females (Fig. 1B). We noticed that the amount of cyclin A2 protein was decreased in virgin but similar in 7 d.p.c. Id2 null glands compared to that in the control glands (Fig. 1B). Immunohistochemically, cyclin A2 and D1 proteins were mainly detected in mammary epithelial cells in both $Id2^{+/-}$ and $Id2^{-/-}$ mice (Fig. 1C). In addition, RT-PCR analyses showed similar expression levels of cdk4 and cdk6 mRNAs between $Id2^{+/-}$ and $Id2^{-/-}$ mammary glands (data not shown). These results indicate that Id2 deficiency does not affect the expression of cyclin D1 or the other cell cycle-related factors examined.

To examine whether Id2 interacts with cyclin D1 at the protein level, cyclin D1 and Id2 were fused to the NF-κB transactivation domain (AD) or GAL4 DNA binding domain (BD) and analyzed with mammalian two-hybrid assays using NIH3T3 cells. As shown in Fig. 2, no apparent interaction was detected between AD-cyclin D1 and BD-Id2 (1.4-fold induction), although robust interactions between Id2 and E12 protein, a dimerization partner of Id proteins (AD-E12(bHLH)/BD-Id2; 115, AD-Id2/BD-E12(bHLH); 30-fold), and between cyclin D1 and cdk4/6 (AD-cyclin D1/BD-cdk4; 58, AD-cyclin D1/BD-cdk6; 71-fold) were detected (Fig. 2 and data not shown). In addition, we also failed to observe an interaction of AD-Id2 with BD-cdk4 or cdk6 (0.6- and 1.0-fold, respectively, Fig. 2 and data not shown). Regarding the switched combinations, namely AD-Id2/BD-cyclin D1 and AD-cdk4/BD-Id2, we could not detect apparent in-

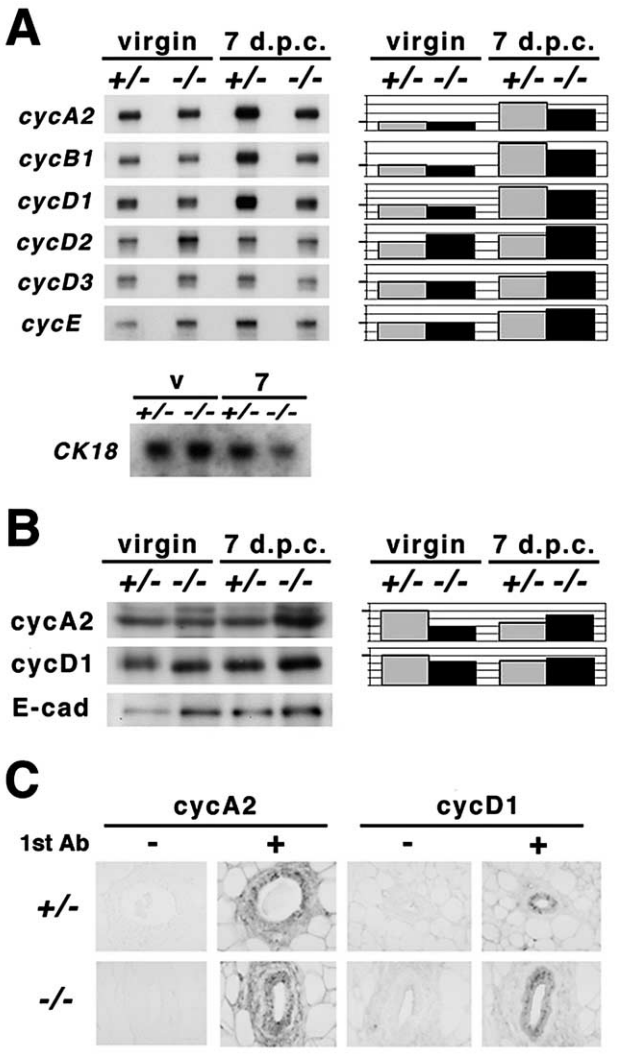

Fig. 1. Normal expression of cyclins in $Id2^{-/-}$ mammary glands. Expression of genes and proteins, indicated on the left, was analyzed in $Id2^{+/-}$ and $Id2^{-/-}$ mammary glands at the indicated d.p.c. Genotypes are indicated at the top of each lane. A: RNase protection assays for gene expression of various cyclins (cycs) in $Id2^{-/-}$ mammary glands. RNAs isolated from 8 to 10 mice at the indicated times were combined and analyzed. Genes and genotypes are indicated on the left and the top, respectively. The results of Northern analysis of CK18 are shown at the bottom. CK18 was used as a marker of epithelial cells. Relative expression levels of cyclins compared to that of CK18 are shown on the right. The relative level of expression in virgin heterozygous mice was designated as 100%. B: Western blot analysis of cyclins A2 and D1 and in $Id2^{-/-}$ mammary glands. E-cadherin (E-cad) was used as an epithelial marker. Protein was extracted from the mammary glands of three individuals, combined and analyzed. Proteins and genotypes are indicated on the left and the top, respectively. Relative expression levels of cyclins A2 and D1 compared with E-cadherin are shown on the right. The relative level of expression in virgin heterozygous mice was designated as 100%. C: Immunohistochemistry for cyclins A2 and D1 in mammary glands. Genotypes and detected cyclins are indicated on the left and the top, respectively. The presence or absence of the respective primary antibodies (1st Ab) are shown in the middle.

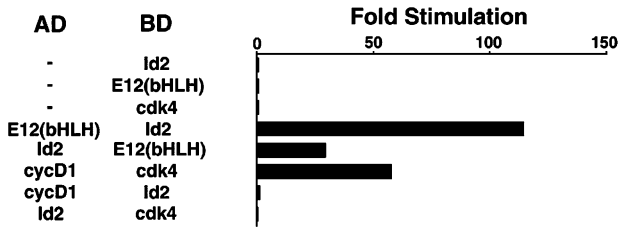

Fig. 2. Lack of interaction of Id2 with cyclin D1 and cdk4 in mammalian two-hybrid assays. AD- and BD-fusion proteins are listed on the left. The results are expressed as fold stimulation relative to the value of each BD-fusion protein with AD alone based on the mean of triplicate samples. Representative data from one of four independent experiments are shown.

teractions. Since we failed to detect the interaction between BD-cyclin D1 and AD-cdk4 in this system (data not shown), BD-cyclin D1 and/or AD-cdk4 may loss their original property due to the formation of fusion proteins. These observations suggest that Id2 does not interact directly with cyclin D1 or cdk4/6.

To investigate the functional relationship between Id2 and cyclin D1 in vivo, we performed crossing experiments of Id2-deficient mice with mammary-specific cyclin D1 transgenic mice that ectopically express human cyclin D1 in mammary epithelial cells under the control of the MMTV 5′ long terminal repeat [6]. This transgene has been reported to be able to rescue the mammary gland phenotype of mice with mutated IKKα [23]. The human cyclin D1 expression in the transgenic mice was confirmed by RT-PCR using a primer set specific to the transgene at 7 d.p.c. and on the first day of lactation irrespective of the Id2 genotype (Fig. 3A). Furthermore, immunohistochemistry confirmed the transgenic protein expression in mammary epithelial cells (data not shown). Thus, the transgene was expressed in mammary epithelial cells as expected.

On the day of parturition, we performed whole-mount analyses of mammary glands of crossed mice. The MMTV-cyclin D1 transgene itself had no effect on the developmental process of the mammary glands in $Id2^{+/-}$ females during pregnancy compared to the lobulo-alveolar development in non-transgenic $Id2^{+/-}$ mammary glands (Fig. 3B). As described previously [5], the mammary glands of non-transgenic Id2 nullizygous mice showed normal side-branching but failed to develop the lobulo-alveolar compartment during pregnancy (Fig. 3B). Similarly the glandular structure was poorly developed in +/Tg; $Id2^{-/-}$ mammary gland after delivery (Fig. 3B). Repeated pregnancies did not improve the situation (data not shown). These results indicate that transgenic expression of cyclin D1 does not rescue the failure of mammary glands of $Id2^{-/-}$ mice and suggest that cyclin D1 is not a downstream factor of Id2 function in the lobulo-alveolar development during pregnancy.

Table 1
Results of BrdU incorporation assay

	$Id2^{+/-}$	$Id2^{-/-}$
+/+	9.8	3.7
	9.5	2.4
+/Tg	7.7	3.7
	8.4	3.3

The results indicate the percentages of BrdU-labeled cells obtained from two individuals in each mouse group. Approximately 1000 nuclei of epithelial cells were examined in each sample taken at 7 d.p.c.

We finally performed BrdU incorporation assays at 7 d.p.c., a time when Id2 null mammary epithelium shows a significant proliferation arrest [5]. In cyclin D1 transgenic $Id2^{+/-}$ mammary glands, the percentage of BrdU-positive epithelial cells was similar to that in non-transgenic Id2 heterozygous glands (Table 1). This observation indicates that ectopic expression of cyclin D1 does not influence the proliferation of normal pregnant mammary epithelial cells. As expected from the results of whole-mount analyses, $Id2^{-/-}$ cells incorporated BrdU less effectively than Id2 heterozygous mammary cells did, irrespective of the presence or absence of the cyclin D1 transgene (Table 1). These data demonstrate that the ectopic expression of cyclin D1 does not overcome the proliferation disturbance or the developmental defect of $Id2^{-/-}$ epithelial cells during pregnancy.

4. Discussion

Several lines of evidence suggest that D-type cyclins and Id proteins share similar features and functions in the regulation of proliferation, which prompted us to investigate the func-

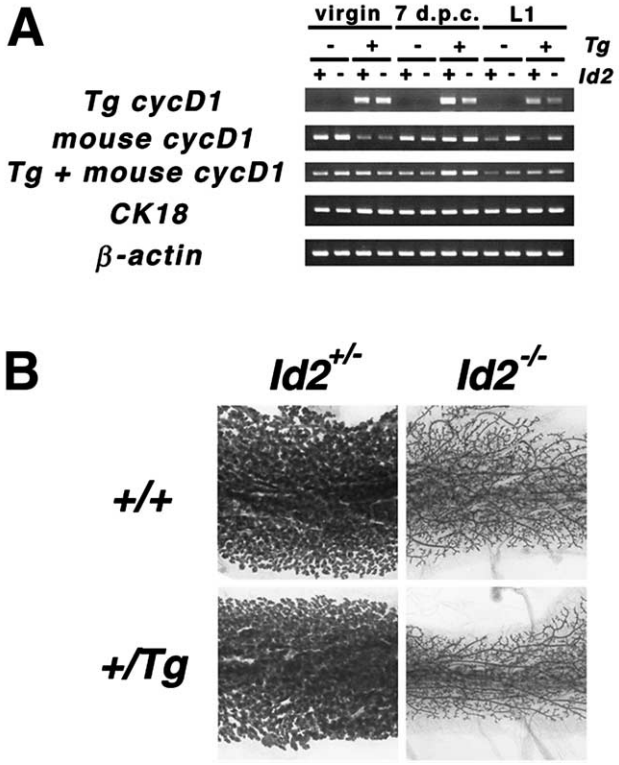

Fig. 3. Forced expression of cyclin D1 does not rescue the failure of mammary gland development caused by Id2 deficiency. A: RT-PCR analysis of expression of the cyclin D1 transgene in $Id2^{-/-}$ mammary glands. The analyses detected the transcript from the transgene, the endogenous murine gene, or both selectively, as indicated on the left. RNA used for the analyses was prepared at the indicated times. L1, the first day of lactation. CK18 was used as a marker of epithelium. β-actin was included as an internal control. Genotypes are indicated at the top. B: Whole-mount analyses of mammary glands of crossed mice. Representative results are shown. Genotypes are indicated at the top (Id2 locus) and the left (MMTV-D1 locus).

tional relationship between cyclin D1 and Id2. Both of these proteins are expressed in a variety of undifferentiated and proliferating cells in vivo and are induced after serum stimulation in cultured cells. Each protein possesses the ability to inhibit cell differentiation and stimulates progression of the cell cycle, especially by its functioning in the phase of G1–S transition in cultured cells [8,13,16,17]. Loss-of-function experiments in mice have demonstrated the requirement for cyclin D or Id for the proliferation of several cell types in vivo, in particular for the proliferation of mammary epithelial cells during pregnancy [4,5,14,15]. They are involved in tumorigenesis as well as in normal cell proliferation. The expression of D-type cyclin or Id proteins is up-regulated in cultured cells in response to oncogenic stimuli [8,13,16]. Cyclin D1 overexpression can be detected in various kinds of primary human cancer lesions, including more than 50% of breast cancers with or without amplification of the gene [24]. Increased expression of Id proteins has been reported in primary human tumors as well as in cultured tumor cells, although alteration of the Id gene loci has not been detected so far [8,13]. Moreover, ectopic expression of cyclin D or Id by transgenesis leads to tumor formation in mice [6,25–27].

MyoD, a bHLH factor of muscle cells, has been reported to bind cdk4 directly. Cdk4 prevents MyoD from binding to DNA, and conversely, MyoD inhibits the activity of cyclin D1/cdk4 [28,29]. This observation suggests that the cyclin D1/cdk4 activity may be regulated by a tissue-specific bHLH factor in the mammary gland. Such a bHLH factor could be a target of Id2. However, we have failed to identify such a factor by degenerate PCR [5] and another group failed to do so by yeast two-hybrid analysis [30]. Based on these facts, we examined here the possibility of a direct interaction between Id2 and cyclin D1 or cdk4/6. However, neither cyclin D1 nor cdk4/6 showed a direct protein–protein interaction with Id2 in mammalian two-hybrid assays. Moreover, Id2 did not modulate the binding affinity of cyclin D1 for cdk4 (data not shown). Therefore, it is not likely that Id2 regulates cyclin D1/cdk4 activity by direct protein interaction.

We demonstrated that the forced expression of cyclin D1 could not substitute for Id2 in the proliferation of mammary cells or in lobulo-alveolar development, suggesting that cyclin D1 is not a downstream factor of Id2 in pregnant mammary epithelial cells. What, then, is the relationship between Id2 and cyclin D1 in mammary gland development? One possibility is that the function of Id2 is separate from that of cyclin D1, but that the functions of Id2 and cyclin D1 might ultimately converge on the promotion of cell proliferation. Cyclin D1 inactivates a pocket domain protein such as retinoblastoma protein (pRb) by phosphorylation, leading to the release of an E2F transcription factor [16–18,31,32]. Since Id2 has been shown to have an antagonistic activity against pocket domain proteins via its direct binding [10], a pocket domain protein seems to be a candidate as the common target. However, the expression levels of E2F-downstream genes, including cyclin D1, cyclin E, DHFR, E2F1 and E2F2 [16,32], are not altered in Id2-deficient mammary glands (this study and data not shown), suggesting that the pRb/E2F pathway is not perturbed in Id2 null mammary cells. Alternatively, it is also possible that cyclin D1 may regulate Id2 activity. For example, cyclin D1 activates cyclin E expression and cyclin E/cdk2 activity through regulation of the pRb/E2F pathway [16,17,32]. The cyclin E/cdk2 complex can phosphorylate the serine residue at the fifth amino acid position in Id2 and weaken the binding affinity of Id2 for an E protein, leading to the progression of cells from G1 to S phase [33].

Recent studies using genetically engineered mice have revealed that there are two categories of genes involved in mammary gland development: one for lobulo-alveolar development (ex. Stat5a and Id2) and the other for predisposition to mammary tumors (ex. c-myc and Wnt) [2,3]. In contrast to cyclin D1 that belongs to both categories, it is currently unclear whether Id2 is involved in mammary tumorigenesis. If Id2 functions as a downstream factor of cyclin D1 directly or indirectly in mammary epithelial cell proliferation, the incidence of cyclin D1-induced mammary tumor formation [6] may be decreased by deletion of Id2. We are now investigating the incidence of such tumors in the crossed mice to examine this possibility.

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