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—Original—

Inhibitory Function of Whey Acidic Protein in the Cell-Cycle Progression of Mouse Mammary Epithelial Cells (EpH4 / K6 Cells)

Kayoko IKEDA[1)], Naoko NUKUMI[1)], Tokuko IWAMORI[1)], Megumi OSAWA[1)], Kunihiko NAITO[1)] and Hideaki TOJO[1)]

[1)]Laboratory of Applied Genetics, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan

Abstract. Although the biological role for whey acidic protein (WAP) in milk has been suggested, its true function is not known. This paper describes evidence for WAP function in the cell-cycle progression of EpH4/K6 (EpH4), mammary epithelial cells *in vitro*. The forced expression of exogenous WAP significantly impaired the proliferation of EpH4 cells, whereas it did not affect that of NIH3T3 cells. Apoptosis was not enhanced in the EpH4 cells with stable expression of WAP (WAP-clonal EpH4 cells). The analyses of BrdU incorporation revealed that forced WAP expression significantly reduced incorporation of BrdU in WAP-clonal EpH4 cells compared with control cells transfected with empty plasmid. Among G1 cyclins, the level expression of cyclins D1 was significantly lower in the WAP-clonal EpH4 cells than in control cells. The inhibitory action of WAP on the proliferation of EpH4 cells was enhanced by the presence of extracellular matrix (ECM), but not by the presence of a single component comprising ECM. The cultured medium of WAP-clonal EpH4 cells inhibited the proliferation of control cells without WAP expression. The present results indicate that WAP plays a negative regulatory role in the cell-cycle progression of mammary epithelial cells through an autocrine/paracrine mechanism.

Key words: whey acidic protein (WAP), Mammary epithelial cells, EpH4 cells, Cell proliferation, Cell cycle, Cyclin Ds

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Whey acidic protein (WAP) was found in the milk of several species including mouse [1], rat [2], camels [3], and rabbits [4]. Recently, it has also isolated from the milk of pigs [5, 6], and three marsupial species, tamer wallaby [7], red kangaroo [8], and brushtail possum [9]. Expression of the WAP gene is regulated by lactogenic hormones, and the level of mouse WAP mRNA in the mammary glands increases thousands-fold between non-lactation and mid-lactation [10]. WAP proteins have a signal peptide [11] and two domain structures that are identified at the four-disulfide core (4-DSC) domain, which is composed of eight cysteine residues in a conserved arrangement [12]. The 4-DSC domain arrangement is not exclusive to the WAP family, and a number of other proteins containing 4-DSC domains have been identified as protease inhibitors [11, 13–16], comprising either single or multiple copies of the 4-DSC domain. Generally, these proteins are secreted and may function as protease inhibitors [17, 18]. Thus, based on the limited sequence identity with a known protease inhibitor, it has been postulated that WAP may be a secreting protease inhibitor [18, 19], and its biological function has been suggested.

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Correspondence: H. Tojo (e-mail: atojo@mail.ecc.u-tokyo.ac.jp)

It has been reported that transgenic mothers in which a mouse genomic WAP transgene is expressed precociously have significantly impaired development of the mammary gland and have failed to lactate [20, 21]. On the other hand, it has been reported that the transgene with WAP promoter or the endogenous WAP is expressed in various exocrine tissues of transgenic female mice [22, 23] or normal lactating mice [24]. These previous findings suggest that WAP may be important as a nutrient in milk for growth, but that it also has some biological function. Nevertheless, to date, there have been few studies that investigated the true function of WAP. Here we report the inhibitory effects of WAP on the proliferation of mammary epithelial cells *in vitro*.

Materials and Methods

Gene constructs

Mouse WAP cDNA was made by reverse transcription with total RNAs that were extracted from mammary glands of C57BL/6J female mice in late pregnancy by TRIZOL Reagent (Gibco BRL, New York, NY, USA) according to the manufacturer's instructions, and subsequently by PCR with the primers 5′-ATG-CGT-TGC-CTC-ATC-AGC-C-3′ (forward) and 5′-GAC-AGG-CAG-GGA-TGG-C-3′ (reverse). A 434-bp segment of WAP cDNA was inserted into a pGEM-T Easy vector (Promega, Madison, WI, USA) and amplified. We determined the DNA sequences of the PCR products and judged the results to indicate a complete cDNA encoding of the full open reading frame of mouse WAP. The pCX-WAP plasmid contained the enhancer of the cytomegalo virus, a chicken β-actin promoter with splicing sequences, WAP cDNA, and the rabbit β-globin 3′ flanking sequences (Fig. 1). EGFP in pCXN-EGFP plasmid (kindly provided by Dr. M. Okabe, Osaka University, Japan) was replaced by the *Eco* RI/*Eco* RI DNA fragment (723 bp). For the transfection of plasmids into cultured cells, pCXN-WAP plasmid, pCXN-EGFP as a marker gene and pCXN plasmid without the coding region as an empty plasmid (mock) were used. pCXN-EGFP plasmid was used for examining the transfection efficiency of plamsids.

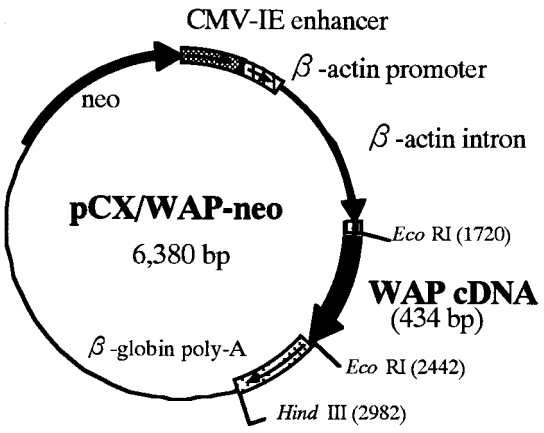

Fig. 1. The gene structure used for transfection into cultured cells. The circular plasmid was transfected into cultured cells for transient expression, and the plasmid linearlized at the *Hind* III site was transfected into the cells in order to generate cells expressing WAP stably. The plasmid in which WAP cDNA was replaced by the EGFP gene (723 bp) was used for examining transfection efficiency of plasmids, and an empty plasmid (mock) was used for transfection as the control.

Cell culture

The cell lines used in this study were EpH4/K6 (EpH4) cells (kindly provided by Dr. W. Birchmeier, Max-Delbritck-Center, Berlin, Germany) [25], and NIH3T3 cells (American Type Culture Collection) as a control for comparison with mammary epithelial cells. The EpH4 cells were grown in a complete medium, Dulbecco's Modified Eagle Medium (DMEM)(Gibco BRL), containing 10% (v/v) heat inactivated FBS, 3.7 g/l of $NaHCO_3$, and 10^5 U/l of penicillin and gentamicin. NIH3T3 cells were grown in a complete medium, containing 10% (v/v) heat-inactivated FBS, penicillin, and streptomycin. All cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 C.

Cell culture on extracellular matrix (ECM)

A 96-well dish was coated with the matrix according to the method of Kuhl *et al.* [26]. The matrices used in this experiment were ECM (basement membrane from Engelbreth-Holm-Swarm tumor, Sigmalaminin), fibronectin, gelatin, laminin, type I collagen, and type IV collagen. Two thousands EpH4 cells per well were plated and cultured for 16 hr in a 96-well dish coated without or with the ECM or the single matrix, and then

transiently transfected with pCX-WAP plasmid, pCX-EGFP or empty plasmid according to the method described in "*Transfection of plasmids*" in this paper. After culturing for another 48 h, the number of live cells was measured as previously described in "*Cell proliferation assay*".

Transfection of plasmids

The plasmids were transfected by means of a Profection Mammalian Transfection System Calcium Phosphate (Promega) according to the manufacturer's instructions. For the establishment of stable plasmid expression, linearized plasmids were transfected into the cells and, after culturing in a complete medium for 2 days, the cells with stable expression of plasmids were selected by passing the culture for 10 days in a complete medium containing G418 (700 μg/ml for EpH4 and NIH3T3 cells).

Cell proliferation assay

Cell-Counting Kit-8 solution (Wako Pure Chemical Co. Ltd., Tokyo, Japan) was added to the medium containing an appropriate number of cells in a 96-well dish reacted at 37 C for 1 h, according to the manufacturer's instructions, and the number of live cells was measured at OD 450 nm with a Microplate Reader (BioRad,Hercules, CA, USA).

Assay of apoptosis in cells

For the assay of apoptosis in the cells, WAP-clonal cells were analyzed according to a previously reported method [27].

Cell-cycle analysis

Incorporation of bromodeoxyuridine (BrdU) into the cultured cells was determined by the recommended protocol of a Cell Proliferation ELISA, BrdU (Roche Ltd, Basek, Switzerland).

RT-PCR

Total RNAs were extracted from the cells with TRIZOL (Gibco BRL) according to the manufacturer's instructions and used for RT-PCR analyses. One microgram of total RNA was denatured and reverse-transcribed into cDNA with SuperScript II (Invitrogen) according to the manufacturer's protocol. PCR was carried out, and the products were then sequenced to confirm the appropriate transcript. The following primer sequences were used for the amplification of target genes. 5′-ATG-CGT-TGC-CTC-ATC-AGC-C-3' (forward) and 5′-GAC-AGG-CAG-GGA-TGG-C-3' (reverse) for WAP cDNA; 5′-CCT-GCA-TGT-TCG-TGG-CCT-CT-3′ and 5′-GAA-AGT-GCG-TTG-TGC-GGT-AGC-3′ for cyclin D1; 5′-AGA-CCT-TCA-TCG-CTC-TGT-GC-3′ and 5′-TAG-CAG-ATG-ACG-AAC-ACG-CC-3′ for cyclin D2; 5′-CCG-TGA-TTG-CGC-ACG-ACT-TC-3′ and 5′-TCT-GTG-GGA-GTG-CTG-GTC-TG-3′ for cyclin D3; 5′-TGA-AGG-TCG-GTG-TCA-ACG-GAT-TTG-GC-3′, and 5′-CAT-GTA-GGC-CAT-GAG-GTC-CAC-CAC-3′ for G3PDH (endogenous control) were used. The quantitative conditions of the PCR reaction were 28 or 31 cycles for 1 min at 94 C, 1 min at 58 C, and 1 min at 72 C. The size of each PCR product was confirmed by electrophoresis with 1% agarose gels in a TAE buffer. RT-PCR products were semi-quantified with NIH image software (NIH, Bethesda, MD, USA. Version 1.60) and were expressed as relative amounts in relation to the PCR product of G3PDH mRNA.

Western blotting

The WAP-clonal cells were cultured in a complete medium without FBS for 2 days after the cells reached at 80% confluence in the medium containing 10% FBS. The cultured medium in the dish was reocvered by centrifugation (10 min, 3,000 rpm), condensed in a Centricon YH-10 (Milipore, Tokyo, Japan), and stored at –80 C until use. WAP protein was detected by Western blotting. Proteins were separated on SDS-PAGE (10% or 15%) gel and transferred to a membrane by semi-dry blotting. The membrane was blocked in Tris-HCl buffered saline (TBS, pH 7.4) containing 5% skim milk for 1 h. After washing with TBS containing 0.1% Tween 20 (TBST), the membrane was incubated with anti-mouse WAP goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 C. After washing with TBST, the membrane was incubated with anti-goat IgG rabbit IgG conjugated to peroxidase (1:5,000) (Jackson Immune Res Lab, Inc., West Baltimore, PA, USA) for 1 h at 4 C. After the membrane was washed with TBST, the specific antibody signal was detected with ECL (Amersham Biosciences, Tokyo, Japan), followed by exposure to X-ray film.

Statistical analysis

Data were analyzed by analysis of variance followed by the Student's *t*-test. Differences

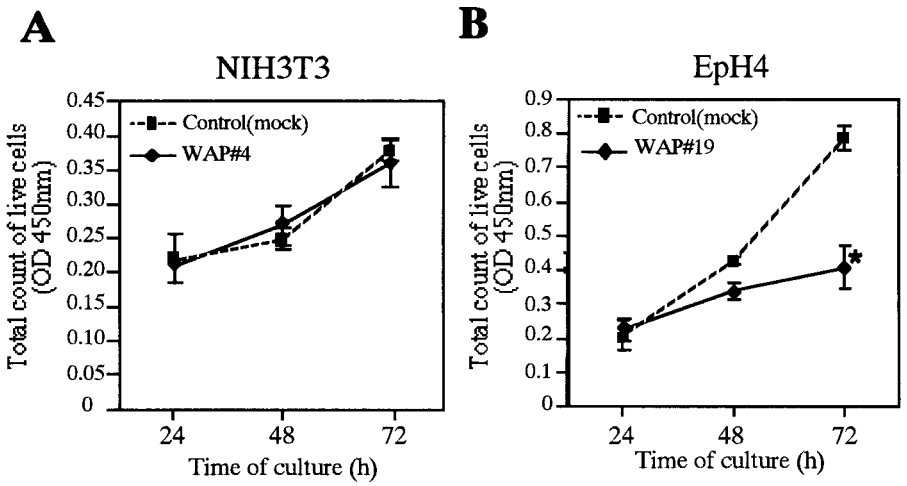

Fig. 2. Time course of proliferation of NIH3T3 cells and EpH4 cells in the presence or in the absence of WAP expression after the start of culture. The proliferation of WAP-clonal EpH4 cells (#19) was significantly impaired at 72 h of culture, but not in WAP-clonal NIH3T3 cells (#4) throughout the culture period. Results represent the mean of 6 experiments and are expressed as OD at 450 nm by means of a Cell Counting Kit-8 (see Materials and Methods for details). The vertical bars indicate standard errors of the SEM (n=6). Control (mock), the cells harboring empty plasmid; #4 and #19, clone number, *, $P<0.05$.

between the mean values were considered statistically significant at $P<0.05$.

Results

Effects of forced WAP expression on the proliferation of EpH4 cells

We established 8 cloned NIH3T3 cell lines and 5 cloned EpH4 cell lines stably expressing the exogenous WAP gene. Using the WAP-clonal NIH3T3 cell line (#4), and the WAP-clonal EpH4 cell line (#19) which showed the highest level of stable WAP expression among the established clones, the effects of WAP expression on the proliferation of these WAP-clonal cells were investigated. There was no difference in the cell proliferation of NIH3T3 cells between the control (harboring empty plasmid) and WAP-clonal cells throughout the culturing period (Fig. 2A), whereas a significant inhibition of cell proliferation was observed in WAP-clonal EpH4 cells at 72 h after the start of culturing compared to the control cells (Fig. 2B).

Autocrine/paracrine action of WAP

To confirm the production of WAP in the WAP-clonal cells and its secretion into the cultured medium, after 2 days-culture, the medium was collected, concentrated, and examined for the presence of WAP by Western blotting. WAP proteins were detected in the medium in which WAP-clonal EpH4 cells were cultured (Fig. 3A). This indicated that exogenous WAP was produced in those cells and was secreted into their cultured medium. The experiments regarding the exchange of cultured medium showed that the cultured medium of WAP-clonal cells significantly inhibited the proliferation of control EpH4 cells at 48 h of culture after medium exchange (Fig. 3B).

Effects of WAP on the cell cycle and expression of cyclin Ds

We examined BrdU incorporation in WAP-clonal cells to determine the effects of WAP on cell-cycle progression. The percentage of BrdU-positive cells was significantly lower ($p<0.05$) in the WAP-clonal EpH4 cells than in control EpH4 cells (mock) and WAP-clonal NIH3T3 cells (Fig. 4). The results of RT-PCR analyses of the expression of G1-phase cyclins, D1, D2 and D3 in WAP-clonal EpH4 cells and control cells are shown in Fig. 5. The expressions of cyclin D1 was significantly lower in WAP-clonal cells than in control cells. The

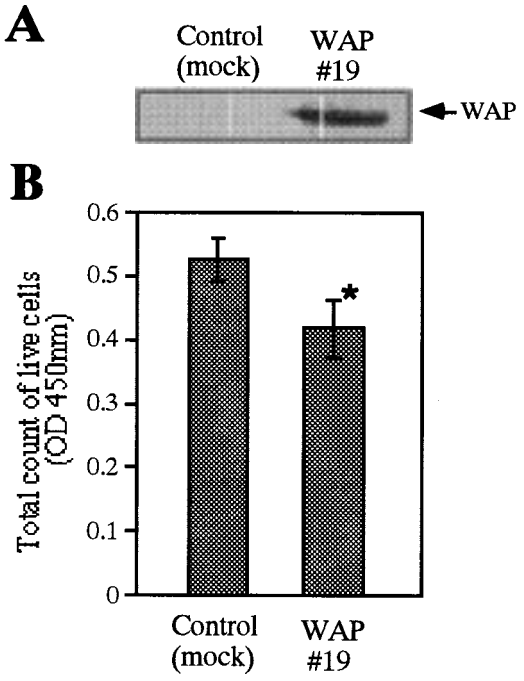

Fig. 3. Evidence for secretion of WAP from WAP-clonal EpH4 cells into medium (A) and its paracrine action on EpH4 cells (B). Control, EpH4 cells transfected with empty plasmid (mock); WAP#19, WAP-clonal EpH4 cells. The vertical bars indicate standard errors of the SEM (n=6). *, P<0.05

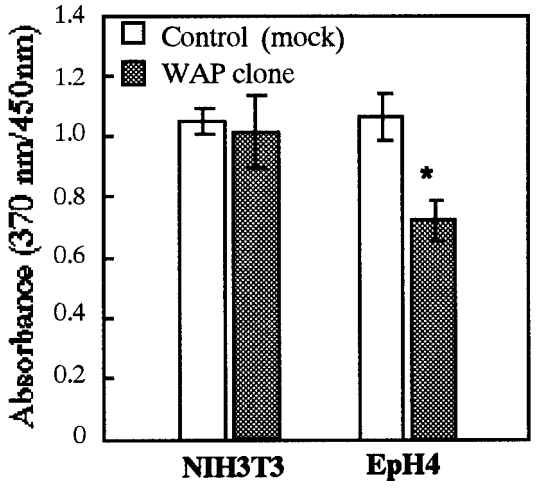

Fig. 4. Incorporation of BrdU in WAP-clonal cells. The values indicate the percentages incorporating BrdU in cells (BrdU/cell) at 4 h after BrdU was added. The incorporation of BrdU was significantly lower (*, P<0.05) in WAP-clonal EpH4 cells than in control EpH4 cells. The vertical bars indicate standard errors of the SEM (n=6).

expressions of cyclin D2 and D3 in WAP-clonal EpH4 cells were not different from those in control cells. By contrast, there was no difference between the level of expression of cyclin Ds in NIH3T3 cells, WAP-clonal cells and control cells (data not shown).

Effects of the presence of matrix on proliferation of EpH4 with WAP expression

To test the effects of ECM and single matrix comprising ECM on the proliferation of EpH4 cells in the presence or in the absence of WAP expression, the cells with or without transient WAP expression were cultured on a matrix-coated and non-coated dish. The number of live cells was counted after culturing for 48 h. The results are shown in Fig. 6. Forced WAP expression in EpH4 cells significantly inhibited cell proliferation as seen in Fig. 3. There was no difference in the proliferation of control cells when they were cultured on an ECM-coated dish and a non-coated dish. By contrast, the proliferation of EpH4 cells was significantly impaired when the cells were cultured on ECM (Fig. 6A). None of five single matrices affected the proliferation of EpH4 cells in the presence of WAP expression (Fig. 6B). When EpH4 cells were cultured on ECM for 6 days, the cells in the absence of WAP expression produced tubular and branched structures (a in Fig. 6C), whereas those in the presence of WAP expression did not produce any tubular structure (b in Fig. 6C).

Discussion

Some insights regarding the possible functions of WAP have been proposed [8, 9, 20, 28–31]. For example, it has been suggested that WAP may be a source of cysteine for hair growth of pouch young because WAP contains a large number of cysteine residues [9]. It has been also suggested that WAP may contribute, *via* milk, to the development of hair in rodents, which are born with minimal fur coverage [7]. On the other hand, Nicholas *et al.* [8] has suggested that WAP may influence the rate of development of the gut of the young *via* milk. Robinson *et al.* [31] have studied WAP/WAP transgenic mice in which WAP is synthesized precociously and have proposed a model for the

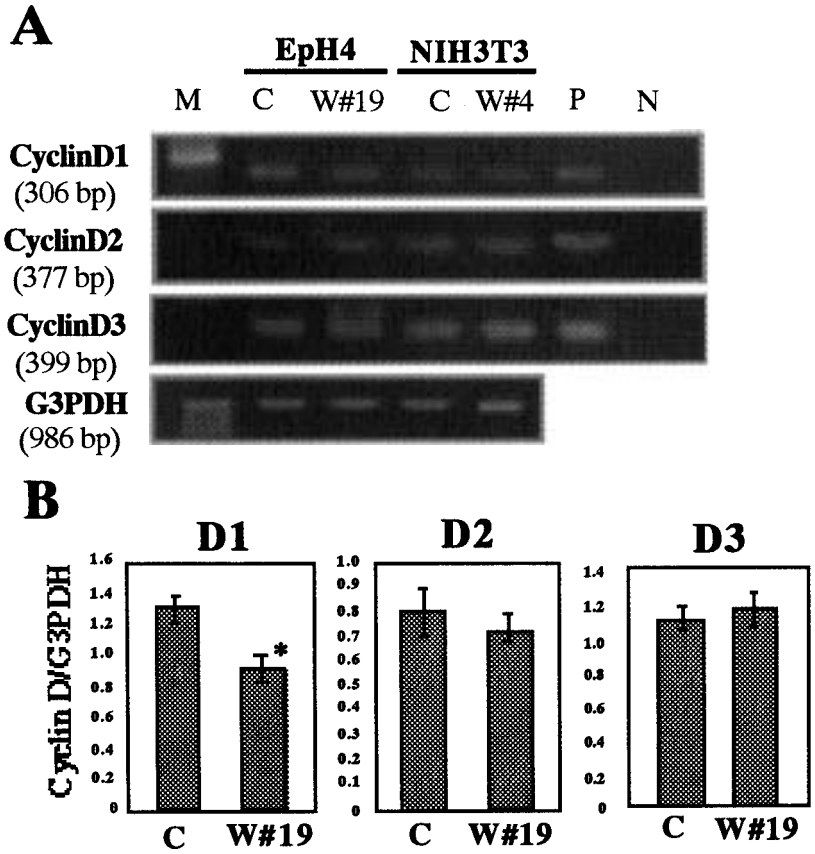

Fig. 5. Expression of G1-phase cyclins (cyclin D1,D2, and D3) in WAP-clonal EpH4 cells. (A), Representative results of RT-PCR analyses. M, molecular size marker; C, cells with empty plasmid; W#19 and W#4, WAP-clonal cells; P, positive control; N,negative control. Values in parenthesis are the size of PCR product. (B), Expression of cyclin Ds in WAP-clonal and control EpH4 cells. There was no difference in the expression of cyclin Ds between WAP-clonal NIH3T3 cells and control cells (data not shown). The vertical bars indicate standard errors of the SEM (n=6). *, $P<0.05$.

regulation of differentiation of mammary alveolar cells where precocious expression of WAP results in terminal differentiation of alveolar cells already at mid-pregnancy, which in turn prevents the alveolar structures from proliferating. We previously reported that transgenic female mice ubiquitously overexpressing the WAP transgene showed impaired lobulo-alveolar development in mammary glands: the proliferation of epithelial cells was markedly impaired, resulting in poor production of β1-casein, which caused agalactia and showed that physiological abnormality other than impaired development of mammary glands occurred in the transgenic mice [32]. These results indicate that WAP action is specific to mammary glands.

In our *in vitro* experiments with cell lines, the forced expression of exogenous WAP significantly inhibited the proliferation of EpH4 cells established from mammary glands, whereas it did not affect that of NIH3T3 cells established from mouse fetal cells. The significant decrease in incorporation of BrdU in WAP-clonal EpH4 cells also indicated that WAP influences the cell-cycle progression of EpH4 cells. RT-PCR analyses of the expression of G1-phase cyclins demonstrated that forced WAP expression in EpH4 cells significantly decreases the expression of cyclin D1, but does not affect the expression of cyclin D2 and D3. D-cyclins are core components of the cell cycle [33] and show significant amino acid similarity [34, 35]. D-cyclins associate with cyclin-dependent kinases CDK4 and

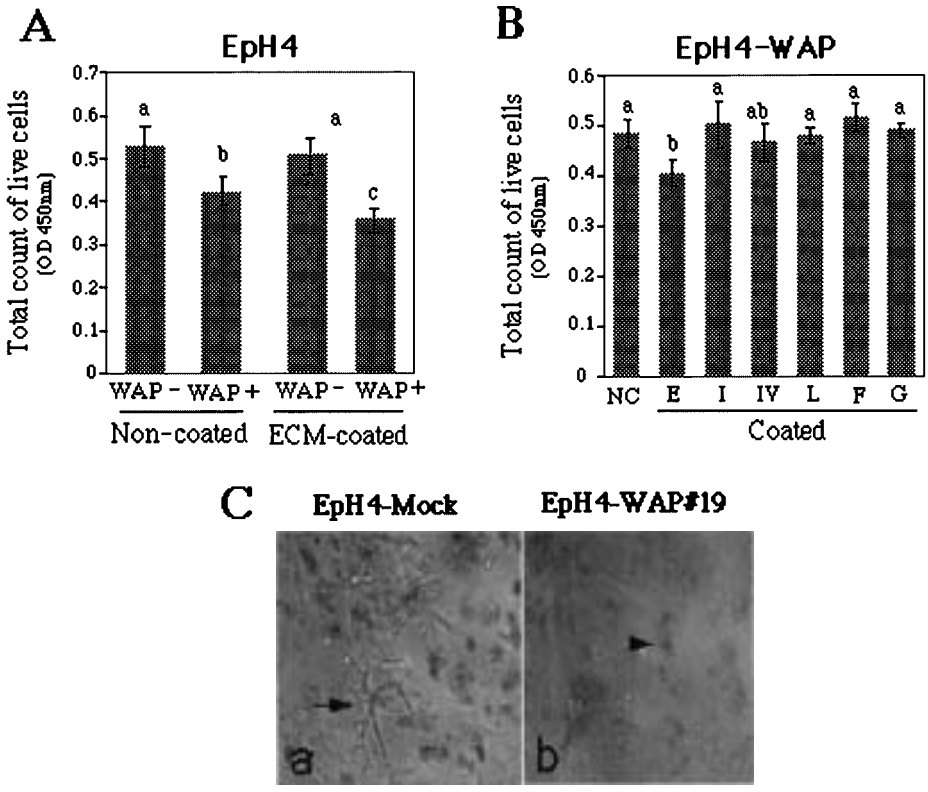

Fig. 6. Effects of ECM and single component of ECM on the proliferation of EpH4 cells with WAP expression. (A), Effect of ECM on the cell proliferation of EpH4 cells in the presence or in the absence of WAP expression. Non-coated, cells transiently transfected with empty plasmid cultured on non-coated dish; ECM-coated, cells on ECM-coated dish; WAP-, cells transiently transfected with empty plasmid; WAP+, cells transiently transfected with WAP-expression vector. (B), Effect of single component of ECM on the proliferation of EpH4 cells in the presence of WAP expression. NC, cells with WAP expression on non-coated dish; Coated, cells with WAP expression on ECM (E), collagen type I (I), collagen type IV(IV), laminin (L), fibronectin (F), and gelatin (G), respectively. Values with different superscript letters are significantly different (Student's *t*-test, $p<0.05$). (C) Effect of forced WAP expression on the morphology of EpH4 cells cultured on ECM. Well-branched tubules were observed in EpH4 cells (EpH4-Mock) with empty plasmid after culturing for 6 days, as indicated by an arrow (a), whereas those (EpH4-WAP#19) stably expressing exogenous WAP showed no branched structures as indicated by an arrow head (b). Original magnification, 300 ×.

CDK6, and drive phosphorylation and subsequent inactivation of the retinoblastoma tumor suppressor, pRB, and pRB-related proteins p107 and p103 [36–38], and in turn causes release or depression of the E2F transcription factors, allowing entry of cells into the S phase [39]. Cyclin D1 is believed to play a critical role in the progression from the G1 to the S phase of the cell cycle. Overexpression of cyclin D1 is seen in a large number of human cancers, including mammary and liver cancer [40–42], and transgenic mice overexpressing cyclin D1 under the control of the mouse mammary tumor virus (MMTV) promoter develop mammary carcinomas [43]. In contrast, adult female mice lacking cyclin D1 fail to undergo the massive epithelial proliferation of mammary glands during pregnancy despite normal levels of ovarian steroid hormones [44]. Thus, cyclin D1 has been found to regulate the epithelial proliferation of mammary glands. The results of our *in vitro* experiments together with those of previous studies of transgenic animals over-expressing exogenous WAP [20, 21, 31, 32, 45] indicate that WAP may play a role in the cell-cycle progression

of mammary epithelial cells during pregnancy and lactation, in particular for the cycle from the G1 to the S phase entry, *via* regulation of cyclin D1 expression.

Based on the limited sequence identity of WAP with known protease inhibitors, it has been thought that WAP may be a protease inhibitor. Proteases and protease inhibitors are thought to regulate tissue formation and remodeling during the development of mammary glands [46, 47]. Therefore, we need to investigate WAP function considering both protease/protease inhibitor interactions and cell-cell interactions in the mammary gland. In the present study, we found that the inhibitory effect of WAP on the proliferation of EpH4 cells was increased by the presence of ECM, but none of the single components comprising ECM affected the proliferation of EpH4 cells with WAP expression.

In conclusion, the results of our study indicate that WAP plays a negative regulatory role in the cell-cycle progression of mammary epithelial cells. The generation of WAP knockout mice should provide a significant tool for determining the true function of WAP *in vivo*.

Acknowledgments

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