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—Original Article—

Decrease of lactogenic hormones induce epithelial-mesenchymal transition via TGFβ1 and arachidonic acid during mammary gland involution

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Abstract. During mammary gland involution, the epithelial mesenchymal transition (EMT) process plays an important role in tissue remodelling and in the termination of milk production. Transforming growth factor β (TGFβ) has been known as a central inducer to EMT and contributor to the mammary gland involution. However, the whole mechanism has accomplished the EMT process in mammary gland is still unclear. Here, we show that arachidonic acid, one of the major products in milk, is new player to control the EMT together with TGFβ during mammary gland involution. Firstly, we observed decrease in *CDH1* (epithelial marker gene) expression and increases in *VIM* and *TWIST1* (mesenchymal marker genes), *TGFB1,* and *PLCG2* (arachidonic acid synthesis gene) at involution. In epithelial cells culture experiments, depletion of lactogenic hormones to mimic the involution induced *TGFβ1* and *PLCG2* expressions. Treatment with arachidonic acid in epithelial cells increased *VIM* and *TWIST1* expressions without decrease of *CDH1* expression, while TGFβ1 decreased *CDH1* and increased *VIM* and *TWIST1*; more importantly, TGFβ1 induced the expression of *PLCG2*, but arachidonic acid did not induce the expression of *TGFB1*. Finally, arachidonic acid accelerated the TGFβ1 increasing *VIM* and *TWIST1* expressions, meanwhile arachidonic acid synthase inhibitor partially blocked the TGFβ1 increasing *VIM* and *TWIST1* expressions. In conclusion, TGFβ1 stimulates arachidonic acid synthesis and the arachidonic acid has a function to postulate the EMT process together with TGFβ1 during mammary gland involution.

Key words: Arachidonic acid, Epithelial-mesenchymal transition, Involution, Mammary gland, TGFβ1

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The mammary gland is a unique organ, providing nutrition and immune protection for a newborn child. Significant structural and functional alternations occur during mammary gland development [1]. During pregnancy, steroid hormones and prolactin stimulate epithelial cell proliferation and differentiation to generate ductal branching and alveolar development, which support milk production during the following lactation period [2]. Lactation is controlled by a sophisticated regulation mechanism taking into account the conflicting requirements to minimize energy cost and maximize the chances for offspring survival. The subtle balance between mother and offspring could influence the lactogenesis and, eventually, mammary gland involution [1]. During involution, the mammary gland undergoes a highly coordinated tissue remodelling process to return to a primitive state in non-pregnancy.

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The epithelial mesenchymal transition (EMT) is an important tissue remodelling process that allows polarised epithelial cells to lose their cell polarity and cell-cell adhesion and gain migratory and invasive properties to become mesenchymal cells [3, 4]. Epithelial cells express high levels of E-cadherin, whereas mesenchymal cells express high levels of N-cadherin, Snail, and Vimentin. Loss of E-cadherin is considered a fundamental event in EMT. There are two types of transcription regulators for E-cadherin expression: Snail1/2 (also known as Slug) and ZEB1/2 can directly bind to the E-cadherin promoter and repress its transcription, whereas factors such as Twist1 can repress E-cadherin indirectly [5, 6]. Therefore, decreased expression of E-cadherin and/or increased expression levels of Vimentin, Snail1/2, ZEB1/2, or Twist1 are molecular markers for EMT.

Transforming growth factor β (TGFβ) is a secreted protein, which regulates cell proliferation, cell differentiation, apoptosis, and other cellular functions [7–10]. There are three mammalian TGFβ isoforms, including TGFβ1, TGFβ2, and TGFβ3. TGFβs play an important local role in mammary gland development [11, 12]. TGFβ expression levels are relatively high during pregnancy but are greatly reduced during lactation because TGFβs inhibit the synthesis of milk protein. During involution, the expression of TGFβs is restored to that during

pregnancy. TGFβ has been known as an inducer and enhancer of EMT and may regulate mammary gland involution though the EMT process [13, 14].

It is believed that inflammation could promote EMT during organ fibrosis and wound healing [15]. The inflammation modulator arachidonic acid and its metabolites have been found in mammary glands and other tissues [16, 17]. Our previous study showed that the arachidonic acid metabolizing enzyme COX-2 has contribution to the mammary gland involution, which indicates the possible function of arachidonic acid or its metabolites in mammary gland involution [18]. Generally, the synthesis of arachidonic acid in cells is depended on two enzymes mediating pathways including phospholipase A2 (PLA2) and phospholipase C (PLC) [19]. The role of arachidonic acid from milk in the mammary gland has been studied over the past decades. While arachidonic acid acts as a milk secretion promoter [20], its contribution to the EMT process during mammary gland involution has not been studied.

The aim of this study was to identify the relationship between TGFβ1 and arachidonic acid in the EMT process during mammary gland involution. We analysed the expression profiles of EMT marker genes, arachidonic acid synthesis genes, and TGFβ1 mRNA during mammary gland development. In addition, we investigated the effects of TGFβ1 and arachidonic acid on expression of EMT marker genes in mammary epithelial cells *in vitro*.

Materials and Methods

Animals

ICR mice were purchased from SLC (Japan SLC, Shizuoka, Japan) and maintained at 23 ± 2°C under a 14-h light schedule (lights on from 0500 to 1900 h). Food and tap water was provided *ad libitum*. The day of the vaginal plug was designated as day 0 of pregnancy (P0). The day of parturition was designated as day 0 of lactation (L0). The pups were rearranged randomly and the equal pups number presented with different mother. Day 10 of lactation, corresponding to the day on which the pups were separated from their mother, was designated as day 0 of forced weaning (W0). Mammary glands were collected from the mice at day 14 of pregnancy (P14); at day 7 of lactation (L7); at days 1 and 2 after forced weaning (involution, I1 and I2); and at days 1, 2, and 3 after putting the pups back with their mother (recovery, R1, R2 and R3) at day I1. Three animals in each stage were used for the experiment. The mammary glands were harvested from the same animals throughout the experimental period. Harvested glands were frozen immediately and stored at –80°C for use in immunofluorescence and real-time quantitative PCR (qRT-PCR) experiments. All experiments with mice were performed according to the guidelines of the Institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology.

Cell culture

Eph4 cells were originally isolated from the mammary tissue of a mid-pregnant Balb/c mouse and routinely maintained in growth media consisting of Dulbecco's Modified Eagle Medium with high glucose (DMEM), 10% foetal bovine serum (FBS), penicillin (50 U/ml), streptomycin (50 µg/ml), amphotericin B (0.125 µg/ml), and insulin (5 µg/ml) [21]. To induce differentiation, confluent cells were incubated for three days with daily replacement of DMEM medium containing lactogenic hormones mix: dexamethasone (1 µM) (Sigma-Aldrich, MO, USA), insulin (5 µg/ml) (Sigma-Aldrich), and prolactin (9 µg/ml) (Sigma-Aldrich). To induce involution, the medium from the differentiated cells was changed to DMEM medium without dexamethasone and prolactin, and cells were incubated for further one day. To investigate the effect of TGFβ1 and arachidonic acid, confluent cells were cultured with TGFβ1 (5 ng/ml) or arachidonic acid (50 µM) or arachidonic acid synthase inhibitor (varespladib, 10 µM) for one day.

Immunofluorescence

Frozen sections (6 µm) of mammary gland tissue were fixed in 4% paraformaldehyde for 10 min, permeabilized, and blocked with 5% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 30 min. Sections were incubated with anti E-cadherin (24E10, Cell Signaling Technology, MA, USA) or anti-vimentin (D21H3, Cell Signaling Technology) antibody 4°C for overnight and with anti-rabbit IgG Alexa Fluor 568 conjugate (Invitrogen, CA, USA) for 1 h at room temperature. These sections were then mounted with ProLong Gold Antifade reagent and DAPI (Invitrogen). Images were captured using a BX-51 immunofluorescence microscope (Olympus, Tokyo, Japan).

Quantitative real-time PCR analysis

Total RNA was extracted from mammary gland tissue or cultured Eph4 cells by using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA was synthesised using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). The oligonucleotide primers for qRT-PCR analysis were designed using the Primer3 program (Table 1). PCR reactions were conducted in a 10-µl volume with Ex Taq Hot Start Version containing SYBR-Green I (Takara Bio) and performed using the chromo4 Real-Time PCR System (Bio-Rad, CA, USA) under the following conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec, and a dissociation protocol. The expression level of each target mRNA was normalized to the reference gene γ-tubulin using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical comparisons were made using Student's *t*-test or one-way ANOVA followed by Tukey's multiple range tests by using Prism 5 software (GraphPad, CA, USA). A P-value of < 0.05 was considered statistically significant.

Results

EMT gene expression during mammary gland development

The expression levels of β-Casein (*CSN2*), E-cadherin (*CDH1*), Twist1 (*TWIST1*), and Vimentin (*VIM*) in the mammary gland during pregnancy, lactation, upon pup removal, and reintroduction of the pups are shown in Fig. 1A. Expression of the gene for the milk protein β-Casein was gradually increased during lactation and reached its peak expression at L7. During involution, when pups were removed from their mother, the *CSN2* expression decreased, but the expression levels recovered when the pups were reintroduced.

Table 1. Oligonucleotide primers used for quantitative real time PCR

Gene name	Forward	Reverse
CDH1	CAAGGACAGCCTTCTTTTCG	TGGACTTCAGCGTCACTTTG
CSN2	AGAGGGATGTGCTCCAGGCTA	TAAGGAGGGGCATCTGTTTG
TWIST1	CCCCACTTTTTGAGGAAGAA	CAGTTTGATCCCAGCGTTTT
VIM	ATGCTTCTCTGGCACGTCTT	CAGTTTGATCCCAGCGTTTT
TGFB1	TGCGCTTGCAGAGATTAAAA	GCTGAATCGAAAGCCCTGTA
PLA2G4A	GCCTCTCTTCACGTGTCTCC	ACCCATCAAGAAATGCAAGG
PLCG2	GGAGCTGAAGACCATCTTGC	CCTAGGATGAACACGGAGGA
TUBG1	GCTGACCAGTGCACGGT	AAACCTGGGGGGCTGGGT

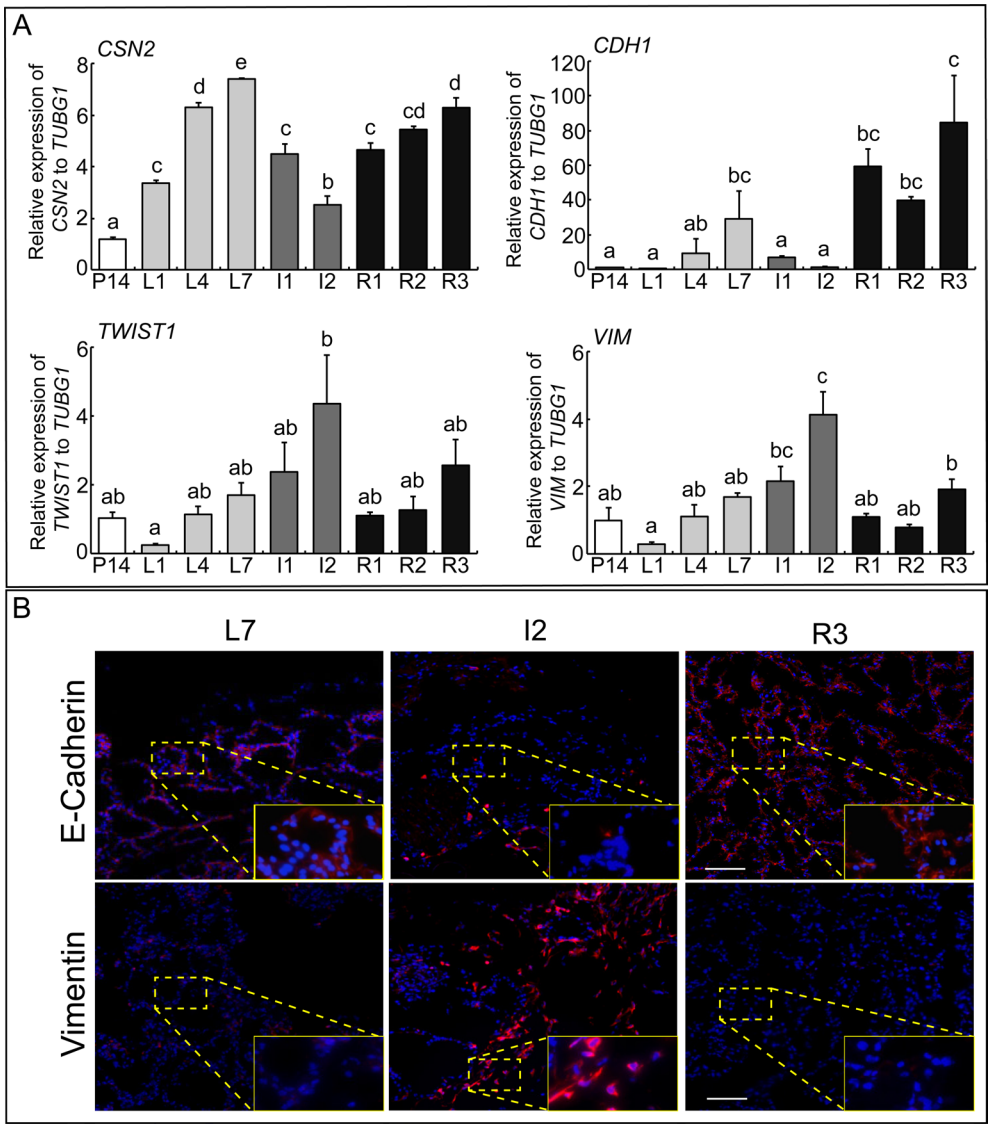

Fig. 1. EMT genes expression during mammary gland development. (A) Real-time PCR of *CSN2*, *CDH1*, *TWIST1* and *VIM* mRNAs in mouse mammary gland at day 14 of pregnancy (P14), day 1, 7 and 10 of lactation (L1, L7 and L10), day 1 and 2 of involution (I1 and I2), and day 1, 2 and 3 of recovery period (R1, R2 and R3). Bars represent means ± SEM for four independent experiments. Different letters indicate significant differences ($P < 0.05$). (B) Immunostained for E-cadherin and Vimentin in the mouse mammary gland at L7, I2 and R3. E-cadherin and Vimentin are shown in red; DAPI is shown in blue. Scale bar, 50 μm.

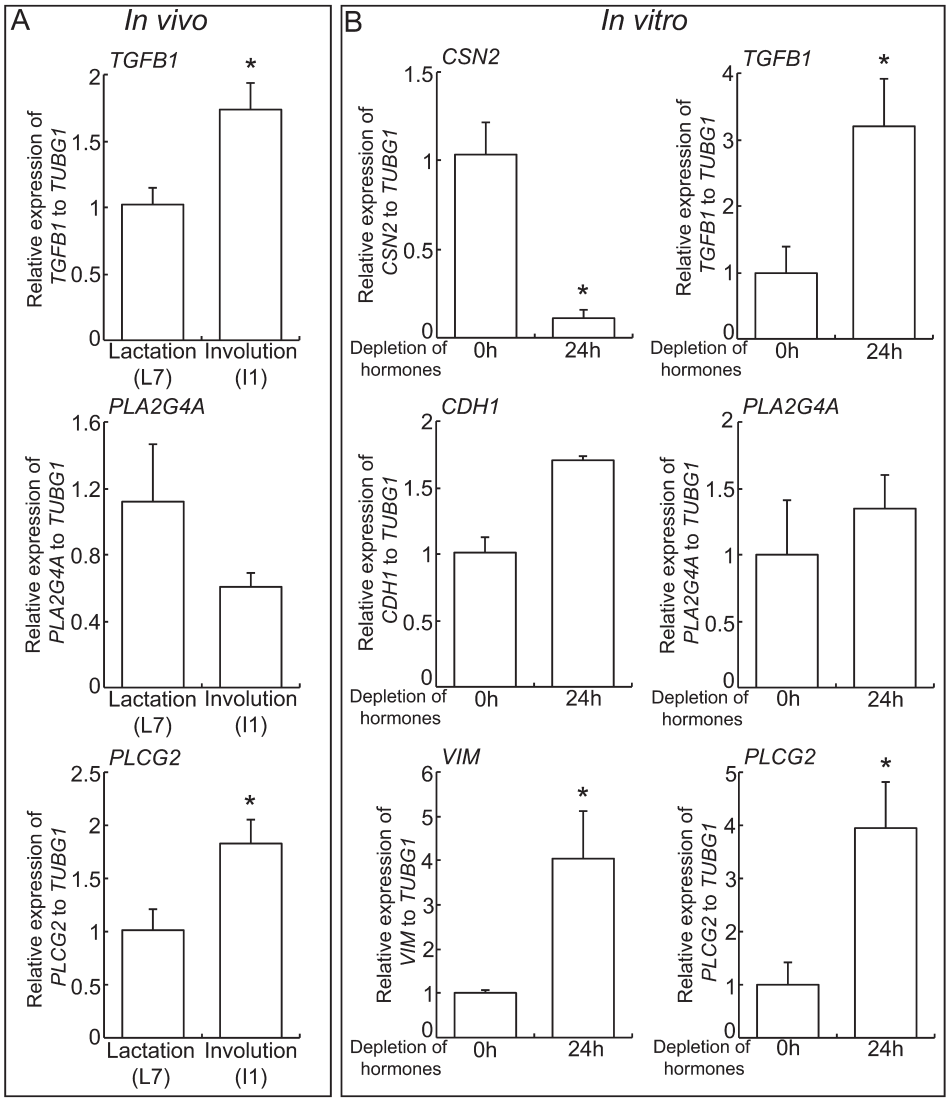

Fig. 2. TGFβ1 and arachidonic acid synthesis genes expression in mammary gland involution. (A) Real-time PCR of *TGFB1*, *PLA2G4A* and *PLCG2* in mouse mammary gland at L7 and I1. Bars represent means ± SEM for four independent experiments. Asterisk indicates significant differences ($P < 0.05$). (B) Real-time PCR of *CSN2, CDH1, VIM, TGFB1*, *PLA2G4A* and *PLCG2* in Eph4 cells incubated in lactogenic hormones-depleted medium for 1 day. Bars represent means ± SEM for four independent experiments. Asterisk indicates significant differences ($P < 0.05$).

The expression pattern of the epithelial marker gene *CDH1* was similar to that of *CSN2,* showing high expression levels during lactation (L4 and L7) and the recovery period (R1, R2, and R3), but low levels in the involution period (I1 and I2). In contrast, the mesenchymal marker genes *TWIST1* and *VIM,* showed constant expression levels throughout the pregnancy and lactation period, a significantly increased expression at day 2 of involution (I2), and sharply decreased expression during the recovery period (Fig. 1A, lower panel). To confirm the manifestation of EMT in the mammary gland, immunofluorescence experiments were conducted for E-cadherin and Vimentin. Similar to the mRNA expression results, high E-cadherin and low Vimentin protein levels were observed in the mammary gland during the lactation and recovery period, while low E-cadherin and high Vimentin levels were detected during involution (Fig. 1B). These results indicate that the EMT is a critical event in mammary gland involution.

TGFβ1 and arachidonic acid synthase gene expression during involution period

To seek the reason for the changes in expression of EMT genes during involution, TGFβ1 and arachidonic acid synthase genes expressions were evaluated by *in vivo* and *in vitro* experiments. The expressions of *TGFB1* and the arachidonic acid synthase *PLCG2*, but not *PLA2G4A*, were increased in the mammary gland tissues during involution period compared to lactation period (Fig. 2A). To investigate a state change from lactation to involution *in vitro*, Eph4,

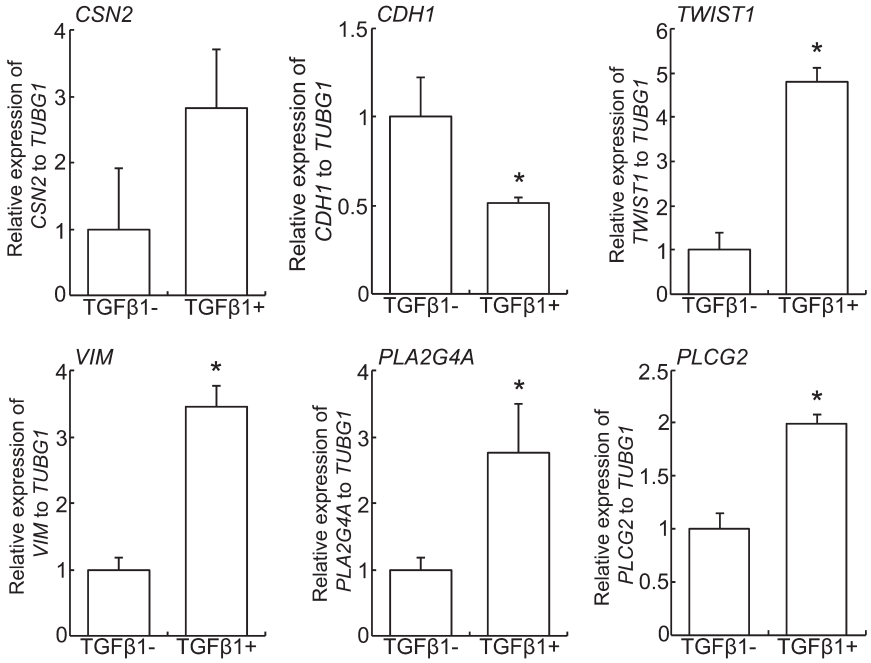

Fig. 3. Effect of TGFβ1 on EMT and arachidonic acid synthesis genes expression. Real-time PCR of *CSN2*, *CDH1*, *TWIST1*, *VIM*, *PLA2G4A* and *PLCG2* in Eph4 cells treated with or without 5 ng/ml human recombinant TGFβ1 for 1 day. Bars represent means ± SEM for four independent experiments. Asterisk indicates significant difference ($P < 0.05$).

mammary gland epithelial cells, were pre-treated with lactogenic hormones mix, insulin, prolactin and dexamethasone, for 3 days to induce cell differentiation observed at lactation, and then further incubated in lactogenic hormones-depleted medium (kept insulin for cell survival) for 1 day to mimic involution [18]. Depletion of prolactin and dexamethasone decreased *CSN2* gene expression and increased *VIM* gene expression even not affected *CDH1* gene expression (Fig. 2B). In this involution model, a significant increase of *TGFB1* and *PLCG2* gene expressions were observed, consistent with the *in vivo* results.

Effect of TGFβ1 on mammary gland epithelial cells

Eph4 cells were treated with recombinant human TGFβ1 for 1 day prior to gene expressions analysis (Fig. 3). The expression of both *TWIST1* and *VIM* was significantly increased in TGFβ1-treated Eph4 cells, meanwhile the expression of *CDH1* was decreased. In addition, the expressions of *PLA2G4A and PLCG2*, encode the consisting proteins of PLA2 and PLC respectively, were significantly increased in TGFβ1-treated Eph4 cells. There was no significant difference in the expression of *CSN2*.

Effect of arachidonic acid on mammary gland epithelial cells

The results of the gene expression analysis in arachidonic acid-treated Eph4 cells are shown in Fig. 4. Treatment of arachidonic acid induced high expression levels of *TWIST1* and *VIM,* but did not affect *CDH1* gene expression. There were no significant differences in the expression of *CSN2* and *TGFB1*.

Interaction between TGFβ1 and arachidonic acid on mammary gland epithelial cells

Eph4 cells were treated with TGFβ1 alone, TGFβ1 with arachidonic acid or TGFβ1 with arachidonic acid synthase inhibitor, and the expressions of *TWIST1*, *VIM* and *CDH1* were analysed by real-time PCR (Fig. 5). Same as in Fig. 3, TGFβ1 stimulated EMT by decreasing *CDH1* and increasing *TWIST1* and *VIM* genes expression. Co-treatment with arachidonic acid and TGFβ1 showed higher increase of *TWIST1* and *VIM* genes expression than TGFβ1 alone, but inconsist increase of the expression of *CDH1* was observed (Fig. 5A). On the other hand, co-treatment with arachidonic acid synthase inhibitor and TGFβ1 increased *CDH1* and decreased *TWIST1* and *VIM* compared to treatment of TGFβ1 alone, indicating that arachidonic acid have a role for induction of EMT by TGFβ1 (Fig. 5B).

Discussion

It has been proposed that mammary gland involution, a rapid and extensive period of tissue remodelling, is accompanied by EMT. In this study, we demonstrated that TGFβ1 and arachidonic acid synthase expression is increased during the involution period in mammary glands and that this increase is observed in cell culture experiments by depletion of lactogenic hormones, prolactin and dexamethasone. Furthermore, TGFβ1 and arachidonic acid could enhance mesenchymal marker gene expression; most importantly, TGFβ1 also increased arachidonic acid synthase genes expression. These results indicate that TGFβ1 and arachidonic acid are both involved in the EMT process during mammary gland involution and

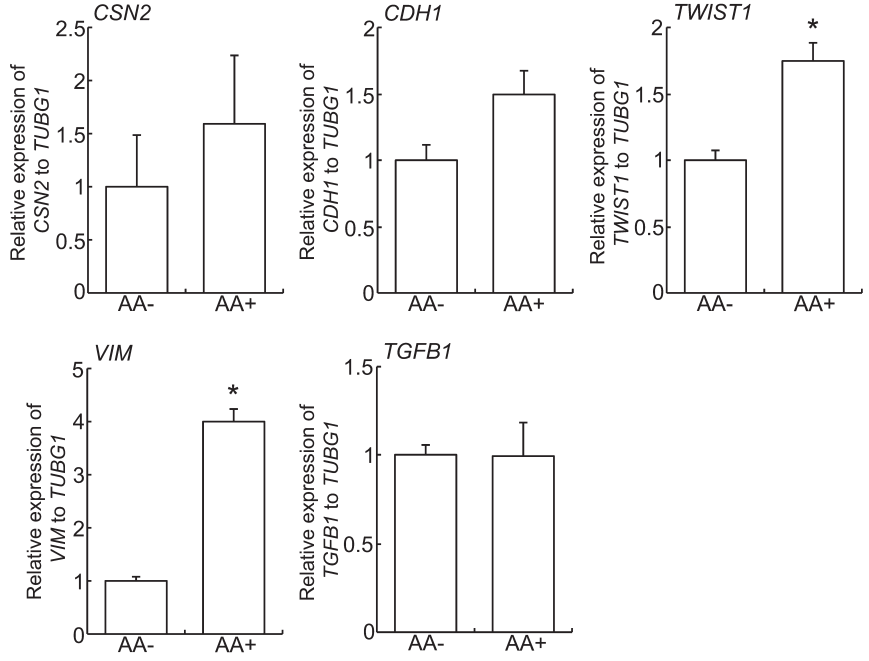

Fig. 4. Effect of arachidonic acid on EMT and TGFβ1 genes expression. Real-time PCR of *CSN2*, *CDH1*, *TWIST1*, *VIM* and *TGFB1* in Eph4 cells treated with or without 50 μM arachidonic acid for 1 day. Bars represent means ± SEM for four independent experiments. Asterisk indicates significant difference ($P < 0.05$). AA, arachidonic acid.

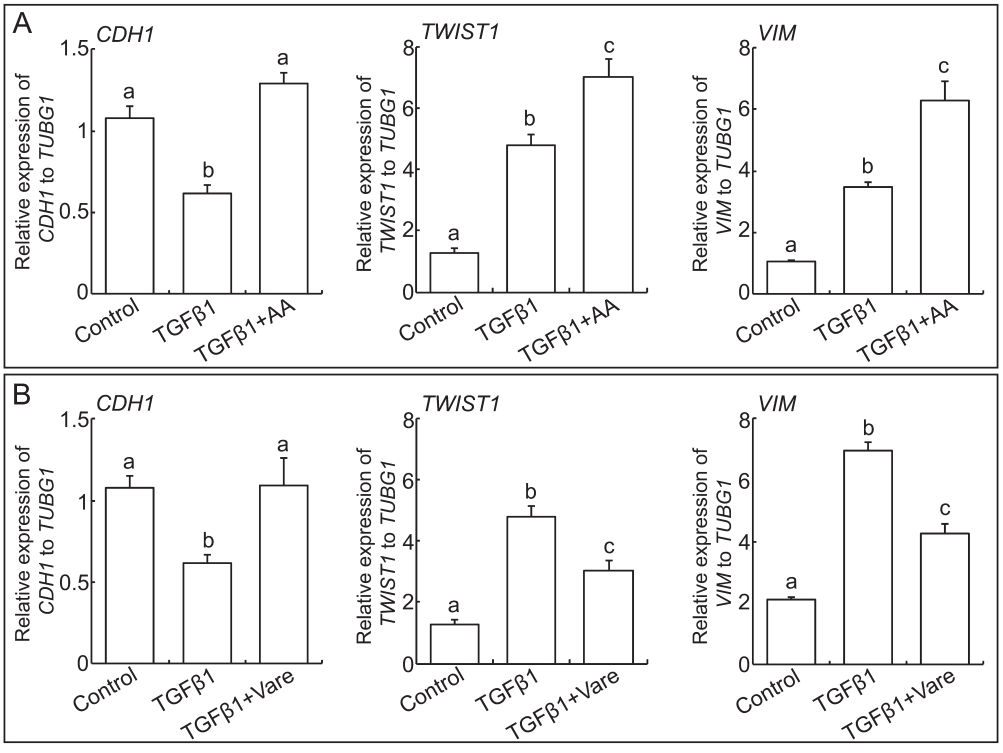

Fig. 5. Interaction between TGFβ1 and arachidonic acid on EMT genes expression. Real-time PCR of *CDH1, TWIST1 and VIM* in Eph4 cells treated with TGFβ1 alone, TGFβ1 with 50 μM arachidonic acid (A) or TGFβ1 with 10 μM Varespladib (B) for 1 day. Bars represent means ± SEM for four independent experiments. The different letters indicate significant difference ($P < 0.05$). AA, arachidonic acid. Vare, Varespladib.

that there is possible interaction between TGFβ1 and arachidonic acid signalling (Fig. 6).

The relationship between a mother and her pups could affect milk production levels in the mammary gland [1]. During lactation, pups demand milk and suck at the mother's nipple, resulting in neural signals that stimulate oxytocin and prolactin secretion from the pituitary gland that increase milk production [22, 23]. Glucocorticoid hormone from adrenal gland postulates the milk production by stimulating prolactin receptor expression in mammary gland epithelial cells [24]. After weaning the fall in these lactogenic hormones and/or milk stasis lead to involution. In this study, EMT genes and *CSN2* expressions were affected by forced weaning, involving the removal of suckling pups from the mother during lactation. In addition, the *in vitro* results showed that depletion of prolactin and dexamethasone in cultured mammary epithelial cells influenced EMT genes expression. These observations suggest that prolactin (and dexamethasone) plays an essential role in regulating EMT genes expression during mammary gland involution, which is consistent with previous reports claiming that prolactin may act as EMT suppressor via decrease TGFβ1 and EMT gene markers expression in breast cancer cells [25, 26]. Following the reduction or depletion of hormones stimulation, TGFβ1 plays a key role in the regulation of the EMT process during mammary gland involution, because increased TGFβ1 gene expression was observed after forced weaning *in vivo* and depletion of prolactin and dexamethasone *in vitro*. TGFβ1 has been shown to biochemically induce EMT for several types of epithelial cells, including normal mammary gland epithelial NMuMG cells, MCF7 human mammary gland tumour cells, and mouse mammary carcinoma cells [27–30]. The mechanism underling the TGFβ signalling pathway involved in the mammary gland involution has been widely investigated [31], however, the interactions between TGFβ and milk composition were remained to be investigated.

The milk compositions include water, carbohydrates, lipids, proteins and minerals, and the bio-active lipid in the milk may exerts molecular functions in mammary gland [32, 33]. The role of arachidonic acid in the mammary gland has been previously investigated with regard to milk secretion [20]. In addition, it has been reported that arachidonic acid can promote the EMT process in normal and cancer mammary epithelial cells [34, 35]. In the present study, arachidonic acid treatment induced *VIM* and *TWIST1* gene expression in Eph4 cells. These observations indicate that increased levels of arachidonic acid and TGFβ are important for the EMT process during mammary gland involution. Interestingly, TGFβ1 could induce the expression of arachidonic acid synthase *PLA2G4A* and *PLCG2*, although arachidonic acid could not induce the expression of TGFβ1 in Eph4 cells. The similar phenomenon was also observed in other cell lines [36, 37]. TGFβ1 activates the PLA2 in cultured rat costochondral chondrocytes [36]. TGFβ1 also activates phospholipase D activity and increases the production of arachidonic acid precursor, diacyl-glycerol (DAG), in lung and kidney epithelial cells [37]. Those results indicate that TGFβ1 signalling pathway may stimulate the arachidonic acid synthesis during the involution period. Furthermore, the co-treatment of TGFβ1 and arachidonic acid could induce the significant higher expressions of mesenchymal markers including *VIM* and *TWIST1* than TGFβ1 alone treatment. Most importantly, the co-treatment with arachidonic acid synthase inhibitor and TGFβ1 suppressed the increase of *VIM* and *TWIST1* expressions by TGFβ1 alone. Taken together, the stimulation of arachidonic acid synthesis by TGFβ1 is important for the accomplishment of EMT process during mammary gland involution period.

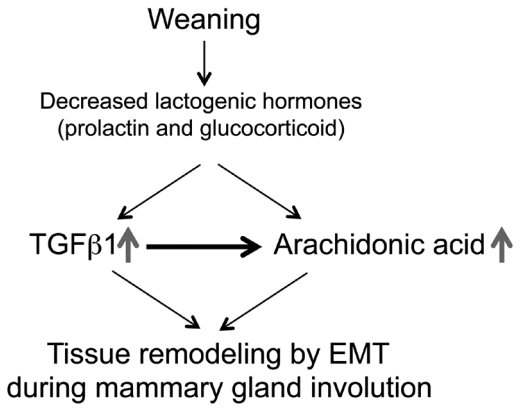

Fig. 6. A schematic representation of the crosstalk between TGFβ1 and arachidonic acid during mammary gland involution. At weaning, a reduction of sucking stimuli decreases lactogenic hormones, prolactin and glucocorticoid, concentration in mother and increase TGFβ1 and arachidonic acid genes expression. TGFβ1 increase of arachidonic acid production in the mammary gland. The interaction between TGFβ1 and arachidonic acid is an important for successful tissue remodelling during mammary gland involution.

In conclusion, the results of this study demonstrate that decreased lactogenic hormones, particularly prolactin and glucocorticoid, initiate EMT process by stimulation of TGFβ1 and/or arachidonic acid synthesis, in addition TGFβ1 itself also stimulates arachidonic acid synthesis. The arachidonic acid and TGFβ1 might accelerate the EMT process by regulating *CDH1*, *VIM* and *TWIST1* genes expression (Fig. 6). Further studies are required to clarify exact molecular mechanisms underlying the interaction between TGFβ1 and arachidonic acid in the EMT process during mammary gland involution.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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