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The Signaling Mechanism of TGF-β_1 Induced Bovine Mammary Epithelial Cell Apoptosis

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ABSTRACT : The present study showed that Transforming growth factor beta 1 (TGF-β_1) can induce apoptosis of bovine mammary epithelial cells. This apoptosis was also observed with phosphorylation of Smad2/3 within 0.5-2 h. Afterwards the signal transferred into the nucleus. Moreover, intracellular free Ca^{2+} concentration was significantly elevated as well as Caspase-3 activated and DNA lysised, thereby inducing the programmed cell death. This signaling pathway of TGF-β_1 was blocked by SB-431542 (10^{-2} μM) via inhibiting ALK-5 kinase activity, which thus reversed the anti-proliferation and apoptosis effect of TGF-β_1 in mammary epithelial cells. These results indicated that TGF-β_1 induced apoptosis of bovine mammary epithelial cells through the ALK-5-Smad2/3 pathway, which plays an important role in inhibiting survival of mammary epithelial cells. Moreover, intracellular Ca^{2+} also played a critical role in TGF-β_1-induced cell apoptosis. (**Key Words :** TGF-β_1, ALK-5, p-Smad2/3, [Ca^{2+}]i, Mammary Epithelial Cell)

INTRODUCTION

It is known that milk cessation of milk production by the mammary gland is the main cause of mammary gland apoptosis. Previous studies in various animals found an over-expressed TGF-β and increased level of mammary gland apoptosis during mammary involution (Faure et al., 2000; Motyl et al., 2001; Wareski et al., 2001) but the level of mammary apoptosis in TGF-β knockout rats was relatively lower during the milk over-store period. The success or failure of a womans lactation and the length of lactation were also dependent on the concentration of TGF-β in human colostrums (Savilahti et al., 2007). It appears that TGF-β is an important inducer of cell apoptosis during mammary involution. Therefore, it would be of great significance to elucidate the signal regulation mechanism of mammary apoptosis induced by TGF-β in improving productivity of dairy animals and the treatment of breast cancer. TGF-β-induced apoptosis of rodent and swine mammary epithelial cells is mainly via a mitochondria-mediated apoptosis pathway (Motyl et al., 2000; Kolek et al., 2003; Xie et al., 2003; Zarzyńska and Motyl, 2005). However, whether TGF-β-induced mitochondria apoptosis within mammary cells occurs through its receptors, for example Smad signaling, is scarcely reported from various animals. Does TGF-β_1 induce mammary cell apoptosis via the ALK-5-Smad2/3 pathway, is intracellular Ca^{2+} involved in the TGF-β_1-induced apoptosis and can ALK-5 kinase inhibitor SB-431542 reverse the effect of TGF-β_1-induced apoptosis? These questions will be addressed in the current study.

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MATERIALS AND METHODS

Epithelial mammary cell culture and treatment

Mammary epithelial cells were cultured in our lab by the technique described by Du et al. (2008). Bovine mammary epithelial cells were cultured in medium containing DMEM/F12 (GIBCO), supplemented with 15% fetal bovine serum (GIBCO). Cell suspension was adjusted to 5×10^4/ml or 1×10^6/ml, and inoculated into a six-well plate or 25 ml flask and cultured for 3 to 5 d. Then the cells were treated in the following groups: i) Treated with 10 ng/ml TGF-β_1 of for 24 h; collected at different time points to detect cell indexes (0 h group without any treatment as control); ii) Treated with SB-431542 (Sigma, #301836-41-9) at different concentrations (0, 10^{-3}, 10^{-2}, 10^{-1} μM) for 1 h, then treated with 10 ng/ml TGF-β_1, followed by collecting at different time points to detect cell indexes (0 h group

without any treatment as control). The SB-431542 solution was prepared in DMSO, diluted using medium before use.

Epithelial mammary cell proliferation detection by MTT assay

Cells were first induced by SB-431542 at different concentrations (0, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} μM) for 1 h, and then treated with 10 ng/ml of TGF-β_1 for 48 h. As described previously (Du et al., 2008), 5 mg/ml MTT was added to cells at a final concentration of 20 μl/well after TGF-β_1 treatment. After culturing for 4 h, the medium was removed and 200 μl of a solution containing 0.1 M HCl and 10% SDS was added to each well, and kept for 12-18 h for complete dissolution. Absorbance at 490 nm was determined by an enzyme micro-plate reader.

Cell apoptosis detection by flow cytometer

Cell *apoptosis* was detected by Annexin V-PI double staining method according to the manufacturers protocol (BD Pharmingen, Cat# 556547).

p-Smad2/3 expression detection by Western blot

As described previously (Cha et al., 2010), the membrane was incubated with p-Smad2/3 polyclonal antibody (1:2,000, Santa Cruz, sc-11769-R) and α-tubulin antibody (1:500, Boster, BM1452), then incubated with the corresponding secondary antibody (1:10,000). The signal was detected using SurpeECL Plus (Applygen, #P1010 and #P1020) as described by the manufacturer. Protein gray scale was detected by Kodak Image Analysis System. The values were defined to be the ratio of the gray scale of p-Smad2/3 protein and α-tubulin protein at the corresponding time points.

p-Smad2/3 expression detection by Immunohistochemistry (IHC)

Immunohistochemical staining was processed using Strept Avidin-biotin Complex (SABC) reagent (Ready to use, Boster, SA1020) kit. P-Smad2/3 polyclonal antibody (1:500, Santa Cruz, sc-11769-R) was used as primary antibody and goat anti rabbit IgG polyclonal antibody was used as secondary antibody. PBS was used as negative control. The positive cell was characterized by yellow particle precipitation in cytoplasm and nucleus under the microscope.

Detection of intracellular [Ca^{2+}]i by flow cytometry

According to the method of Chen et al. (2003), cells were collected and centrifuged at 1,000 r/min for 5 min. Supernatant was removed and the precipitate was resuspended by adding 300 μl PBS (Ca^{2+}, Mg^{2+}). Subsequently, 2 μl of 1 mM Fluo3-AM (Invitrogen, #F1242) was added followed by shaking and incubating at 37°C for 30 min. Then centrifuged at 1,000 r/min for 5 min. Afterwards the supernatant was removed and the precipitate was resuspended with 1 ml of PBS (Ca^{2+}, Mg^{2+}). The concentration of Ca^{2+} was detected by flow cytometry.

Caspase-3 activity analysis

Caspase-3 activity was measured with a Caspase-3 Colorimetric Assay Kit (CaspACE_Assay system, Promega) according to manufacturers instructions. Caspase-3 enzymatic activity was calculated by rate of Caspase-3 shearing substrates/weight of protein (μM/min/mg protein).

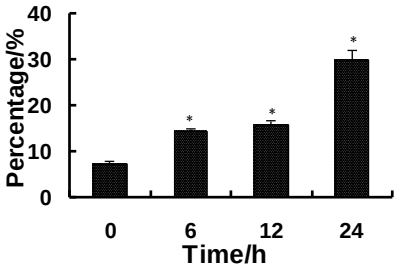
Data analysis

There were 3 to 12 repeats in each group, and 3 independent repeats in each experiment. One-way ANOVA (analysis of variance) and LSD were used to assess statistically significant differences between control and treatment group by SPSS 11.0.

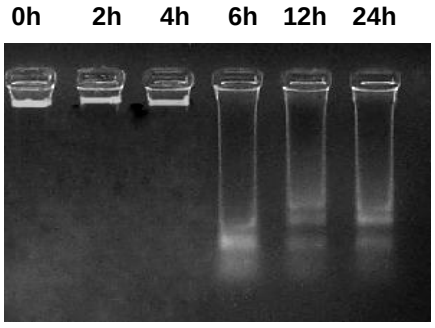
RESULTS

TGF-β_1 activated Smad2/3 phosphorylation and transmitted the signal to induce the apoptosis of bovine mammary epithelial cells

Figure 1a shows that the ratio of cell apoptosis increased with lengthening of TGF-β_1 treatment time, and



(a)



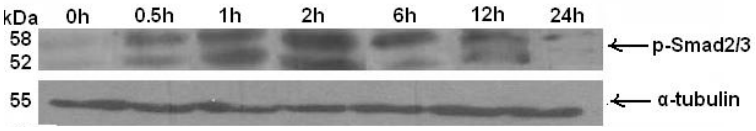
(b)

Figure 1. The variation of mammary epithelial cells treated with TGF-β_1. (a) The apoptosis ratio of mammary epithelial cells treated with TGF-β_1; (b) The DNA fracture of bovine mammary epithelial cells treated with TGF-β_1. * Indicates a significant difference between two groups ($p<0.01$).

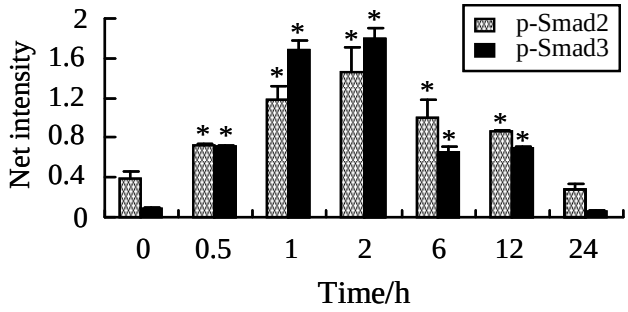
cell apoptosis at 6 h, 12 h, and 24 h groups was significantly higher than the control group ($p<0.01$), suggesting the mammary gland epithelial cell's apoptosis was dependent on TGF-β_1 treatment time. It can be seen in Figure 1b that DNA cleavage appeared when epithelial cells were treated for 6 h, 12 h and 24 h (Figure 1b), The phosphorylation levels of Smad2/3 significantly increased after mammary epithelial cells were treated by TGF-β_1 (10 ng/ml) for 0.5 to 2 h and reached peak at 2 h (Figure 2a) while the phosphorylation levels of all groups were significantly higher than the control except for the treated 0 h and 24 h groups ($p<0.01$) (Figure 2b). The result by Immunohistochemical analysis was identical with the result of Western blot that obvious p-Smad2/3 transferring into nucleus was observed when epithelial cells were treated for 2 h (Figure 2c).

TGF-β_1 induced bovine mammary epithelial cell apoptosis was dependent on intracellular Ca^{2+} and activity of Caspase-3

The intracellular Ca^{2+} level started to increase when mammary epithelial cells were treated with TGF-β_1 for 3 h, and reached peak at 6 h and decreased at 12 h, The levels of all experimental groups were significantly higher than the control group ($p<0.01$, Figure 3a). Meantime the intracellular activity of Caspase-3 increased when



(a)



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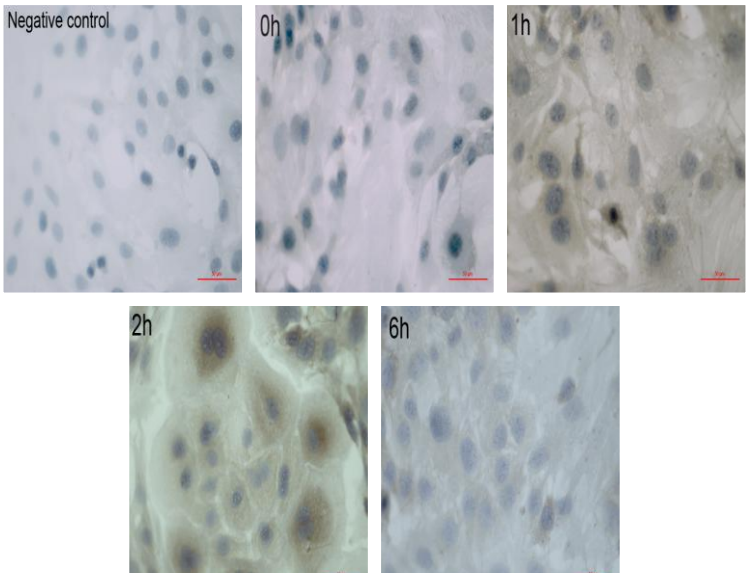

(c)

Figure 2. The expression of Smad2/3 within mammary epithelial cells treated with TGF-β_1. (a) The p-Smad2/3 express changes in mammary epithelial cells treated with TGF-β_1. (b) The expression of p-Smad2/3 within mammary epithelial cells treated with TGF-β_1. (c) The expression and location of p-Smad2/3 within mammary epithelial cells treated with TGF-β_1. * Indicates a significant difference between two groups ($p<0.01$).

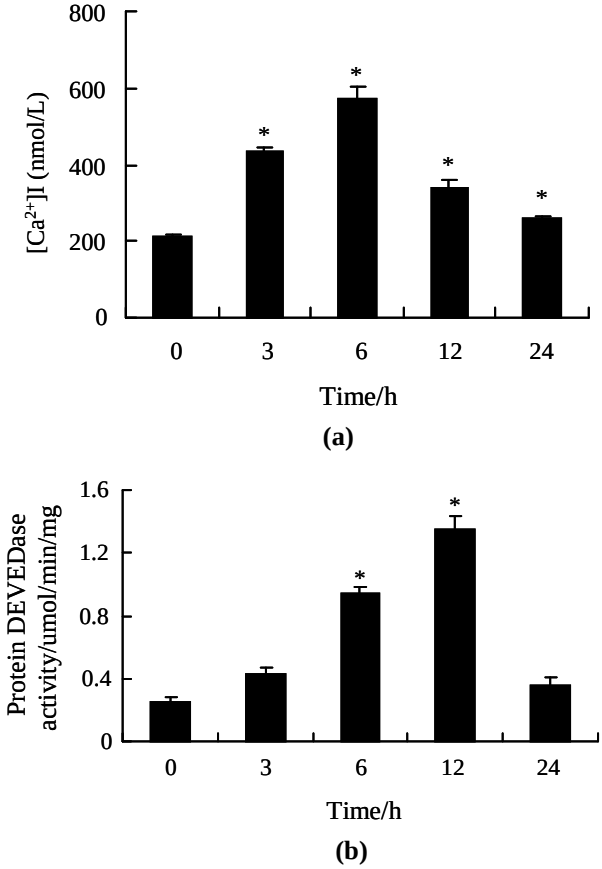

Figure 3. The variation of intracellular $[Ca^{2+}]i$ and activity of Capase-3 treated with TGF-β_1. (a) The concentration changes of intracellular Ca^{2+} within mammary epithelial cells treated with TGF-β_1. (b) The activity changes of Capase-3 within mammary epithelial cells treated with TGF-β_1 at different durations (0, 3, 6, 12, 24 h). * Indicates a significant difference between treated groups and control ($p<0.01$).

mammary epithelial cells were treated for 6 h and reached peak at 12 h. The activity of Capase-3 in two groups (6 h group and 12 h group) was also significantly higher than that of the control group ($p<0.01$) (Figure 3b). These results indicated that TGF-β_1-induced bovine mammary epithelial cells apoptosis was dependent on the intracellular Ca^{2+} and activity of Caspase-3.

SB-431542 reversed anti-proliferation and apoptosis-accelerating effect of TGF-β_1 in mammary epithelial cell

The result showed that cell proliferation reached the highest level when cells were treated with 10^{-2} μM of SB-431542, and it was significantly different between the treated group, control group and DMSO vector group ($p>0.05$) (Figure 4a). The cell apoptosis ratio showed no difference between 10^{-2} μM SB431542 treatment and control ($p>0.05$), but it was significantly different between 0 μM group and others groups ($p<0.01$) (Figure 4b). Our results also showed that 10^{-2} μM SB-431542 blocked the

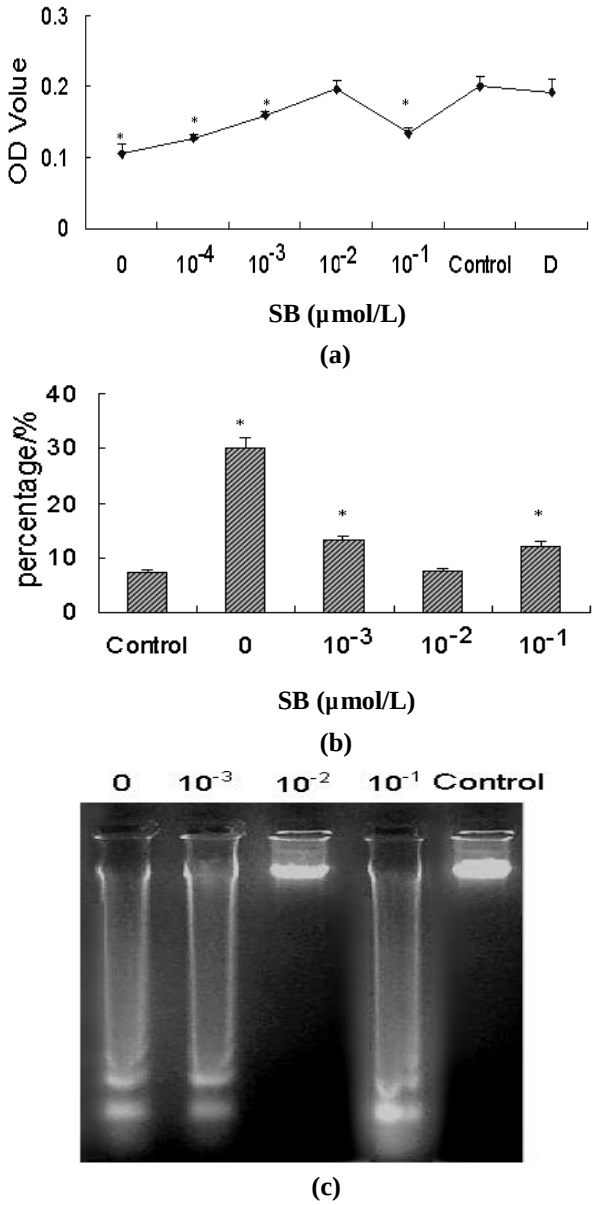

Figure 4. SB-431542 blocked the proliferation and apoptosis induced by TGF-β_1. The mammary epithelial cells were treated with SB-431542 of different concentrations (0, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} μM) for 1 h, then treated with 10 ng/ml of TGF-β_1. (a) The proliferation of mammary epithelial cells detected by MTT method. The group without any treatment is defined as the control, and D is DMSO blank vector group. (b) The apoptosis ratio of mammary gland epithelial cells detected by flow cytometry. * Indicates a significant difference between groups ($p<0.01$). (c) The detection of DNA integrity in mammary gland epithelial cells.

TGF-β_1-mediated DNA degradation (Figure 4c).

SB-431542 blocked the effect of TGF-β_1 on intracellular Smad2/3 phosphorylation, intracellular Ca^{2+} and the activity of Caspace-3 in mammary epithelial cells

Smad2/3 phosphorylation levels gradually decreased as SB-431542 concentration increased, in a dose-dependence

manner (Figure 5a). Smad2/3 phosphorylation levels of 10^{-2} μM group was significantly lower than that of group treated with TGF-β_1 for 2 h ($p<0.01$), but there was no difference compared with the control group ($p>0.05$) (Figure 5b). These results indicated that SB-431542 inhibited TGF-β_1-mediated Smad2/3 phosphorylation which was confirmed by immunohistochemistry (Figure 5c). $[Ca^{2+}]i$ (Figure 5d) and Caspase-3 activity (Figure 5e) of 10^{-2} μmol/L SB-431542 group was also lower than that of the group treated with TGF-β_1 ($p<0.01$). Taking these results together, it suggests that SB-431542 blocked TGF-β_1-induced epithelial cell apoptosis through inhibiting the intracellular Smad2/3 phosphorylation levels, $[Ca^{2+}]i$ levels and the activity of Caspace-3.

DISCUSSION

TGF-β_1 induced apoptosis of bovine mammary epithelial cells cultured *in vitro*, but the detailed mechanism involved is not clear. Here we showed that the Smad2/3 phosphorylation level significantly increased when epithelial cells were treated with TGF-β_1 for 0.5 to 2 h and p-Smad2/3 was also transferred into the nucleus, indicating that TGF-β_1-induced mammary epithelial cell apoptosis was dependent on transfer signals by Smad protein. It has been found that there was an obvious Smad-DNA complex after MEC was treated with TGF-β_1 for two hours (Gajewska et al., 2005) and that the TGF-β_1 signal transmitting through Smad2/3 was finished within few minutes after the combination and activation of TGF-β_1 and its receptor.

Ca^{2+} plays an important role in signal transduction of cell apoptosis. There are three main pathways involved in cell apoptosis: mitochondria pathway, Endoplasmic Reticulum (ER) pathway and death receptors pathway, of which Ca^{2+} is the hub (Walter and Hajnoczky, 2005). Under certain stimulation, ER releases its stored Ca^{2+} causing the elevation of cytosol Ca^{2+}, which is further obtained by mitochondria. The Ca^{2+} overloading in mitochondria leads to mitochondria damage, leading to cytochrome C releasing into the cytosol, which directly activates the cascade of Caspase-3 involved apoptosis and inhibits the expression of BCL-2, IAP, and therefore initiates cell apoptosis (Breckenridge et al., 2003; Kadenbach et al., 2004; Walter and Hajnoczky, 2005; Lamb et al., 2006). Death receptor Fas-induced apoptosis also functions via increasing cytosol Ca^{2+} concentration (Ashkenazi and Dixit, 1998). It was

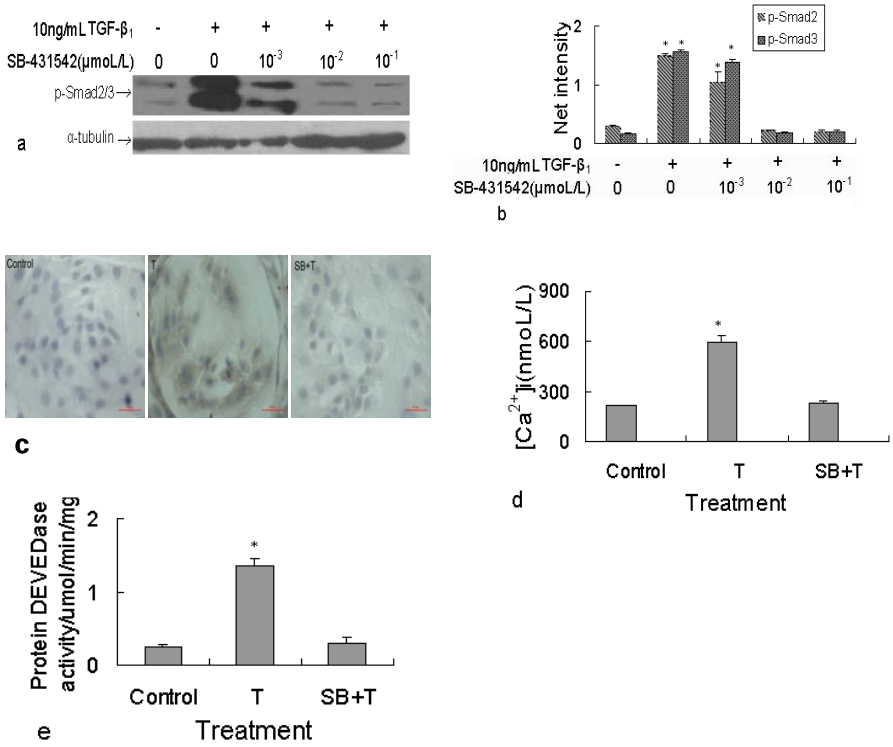

Figure 5. The variation of phosphorylation of Smad2/3, concentration of Ca^{2+} and activity of Caspace-3 in mammary epithelial cells treated with SB-431542. (a) The express changes of p-Smad2/3 treated with SB-431542 at varied concentration (0, 10^{-3}, 10^{-2} and 10^{-1} μM) for 1 h, then treated with 10 ng/ml of TGF-β_1 for 2 h. (b) The expression changes of p-Smad2/3 treated with SB-431542. (c) The expression changes of p-Smad2/3 of different treatments: control, TGF-β_1 2 h treatment, SB+TGF-β_1 2 h treatment. (d) The intracellular Ca^{2+} of different treatments: control, TGF-β_1 6 h treatment, SB+TGF-β_1 6 h treatment. (e) The activity of Capase-3 of different treatments: control, TGF-β_1 12 h treatment, SB+TGF-β_1 12 h treatment. * Indicates a significant difference between two groups ($p<0.01$).

found in the present study that Ca^{2+} significantly increased when epithelial cells were treated with TGF-β_1 for three hours and the activity of Capase-3 increased significantly when treated for six hours, indicating that TGF-β_1 induced epithelial cell apoptosis in a Ca^{2+} and Caspase-dependent manner. Bad was activated during TGF-β_1-induced apoptosis, but the expression of Fasl decreased (Song et al., 2000) and the expression of apoptosis-promoting proteins of Bcl-2 family increased in mammary tissue during involution. Therefore, it is likely that TGF-β_1 induced epithelial cell apoptosis through the mitochondria and ER pathway instead of the death receptors pathway.

The I type receptors of TGF-β include ALK-1 and ALK-5, which transduct different Smad signals respectively within vascular endothelial cells. SB-431542 enhances the the growth and integrity of epithelial cells derived from embryonic stem cells. It was proven that the ALK-5-Smad2/3 pathway inhibits cell proliferation and differentiation, but the ALK-1-Smad1/5 pathway is reversed (Goumans et al., 2002; Watabe et al., 2003). It was shown that SB-431542 retarded activation of ALK-5- Smad2/3 pathway and its signal transduction, inhibited the Ca^{2+} level and the activity of Capase-3 through inhibiting ALK-5 kinase activity, which further blocked the effect of TGF-β_1 on anti-proliferation and apoptosis-accelerating of bovine mammary epithelial cells, and protected DNA integrity and cell survival. It was also found that SB-431542 can promote the growth and integrity of epithelial cells derivated from embryonic stem cells (Watabe et al., 2003). These results indicated that TGF-β_1 may induce mammary epithelial cell apoptosis through the ALK-5- Smad2/3 pathway, which plays a role in inhibiting proliferation and survival of mammary epithelial cells. Transforming growth factor-beta1 causes pulmonary microvascular endothelial cell apoptosis via ALK5 (Lu et al., 2009). It was also suggested that the changes of TGF-β_1-induced intracellular Ca^{2+} and Caspases-3 activity were regulated by ALK-5 kinase, which is a subsequent event following the ALK-5- Smad2/3 pathway in terms of time effect. Therefore, it is suggested that TGF-β_1 induced mitochondria and Endoplasmic Reticulum pathway within epithelial cells via the ALK-5-Smad2/3 pathway. It is also proven that the apoptosis of epithelial cells is an important factor in causing mammary gland degradation. But the molecular mechanism involved in TGF-β_1-regulated Ca^{2+} concentration alterations via ALK-5-Smad2/3 and Ca^{2+} activating apoptosis-regulating factors remains to be further explored. Recently, the signal pathway of TGF-β has become a target for cancer treatment (Lv et al., 2010; Ungefroren et al., 2011), and inhibiting TGF-β_1 I-type receptor kinase selectively can inhibit the proliferation and transfer of mammary cancer cells of mouse, and activating TGF-β_1 I-type receptors (ALK-5) kinase can improve survivability of mammary epithelial cells and promote the progress of breast tumor (Ge et al., 2006; Muraoka-Cook et al., 2006). It was shown in this study that SB-431542 could block the signal pathway of TGF-β, therefore SB-431542, a TGF-β_1 I-type receptors kinase inhibitor, is a potential new drug in treating metastatic breast tumors. It was also found that the phosphorylation level of Smad3 was slightly higher than that of Smad2 after treated with TGF-β_1 for 0.5 to 2 h, whereas the phosphorylation level of Smad3 was slightly lower than that of Smad2 after treatment with TGF-β_1 for 6 h (Figure 2a and 5a), which suggested that Smad2 and Smad3 may play different roles when transmitting TGF-β_1 signals in cells. Smad3 may be more important than Smad2 in TGF-β induced epithelial cells period retard (Brown et al., 2007). Thus, it would be important to elucidate the mechanism of the mammary apoptosis mediated by TGF-β and understand how Smad2 and Smad3 regulate signal pathway of TGF-β differently.

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