Journal of Cellular Biochemistry 104:1647–1659 (2008)

TGF-β Inhibits Prolactin-Induced Expression of β-Casein by a Smad3-Dependent Mechanism

Wen-Jun Wu,[1] Chin-Feng Lee,[2] Chung-Han Hsin,[3,4,5] Jyun-Yi Du,[2] Tsai-Ching Hsu,[2] Ting-Hui Lin,[6] Tsung-You Yao,[2] Cheng-Hsieh Huang,[1] and Yi-Ju Lee[2]*

[1]Institute of Medical and Molecular Toxicology, Chung Shan Medical University, Taichung, Taiwan, ROC
[2]Institute of Immunology, Chung Shan Medical University, Taichung, Taiwan, ROC
[3]Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan, ROC
[4]Department of Medicine, Chung Shan Medical University, Taichung, Taiwan, ROC
[5]Department of Otolaryngology, Chung Shan Medical University Hospital, Taichung, Taiwan, ROC
[6]Department of Biomedical Sciences, Chung Shan Medical University, Taichung, Taiwan, ROC

Abstract Transforming growth factor-β (TGF-β) is a multifunctional growth factor, affecting cell proliferation, apoptosis, and extracellular matrix homeostasis. It also plays critical roles in mammary gland development, one of which involves inhibition of the expression of milk proteins, such as β-casein, during pregnancy. Here we further explore the underlying signaling mechanism for it. Our results show that TGF-β suppresses prolactin-induced expression of β-casein mRNA and protein in primary mouse mammary epithelial cells, but its effect on protein expression is more evident. We also find out that this inhibition is not due to the effect of TGF-β on cell apoptosis. Furthermore, inhibition of TGF-β type I receptor kinase activity by a pharmacological inhibitor SB431542 or overexpression of dominant negative Smad3 substantially restores β-casein expression. By contrast, inhibition of p38 and Erk that are known to be activated by TGF-β does not alleviate the inhibitory effect of TGF-β. These results are consistent with our other observation that Smad but not MAPK pathway is activated by TGF-β in mammary epithelial cells. Lastly, we show that prolactin-induced tyrosine phosphorylation of Jak2 and Stat5 as well as serine/threonine phosphorylation of p70S6K and S6 ribosomal protein are downregulated by TGF-β, although the former event requires considerably long exposure to TGF-β. We speculate that these events might be involved in repressing transcription and translation of β-casein gene, respectively. Taken together, our results demonstrate that TGF-β abrogates prolactin-stimulated β-casein gene expression in mammary epithelial cells through, at least in part, a Smad3-dependent mechanism. J. Cell. Biochem. 104: 1647–1659, 2008.

Key words: mammary gland; prolactin; β-casein; TGF-β; Smad3

A unique feature of the mammary gland is that its development occurs primarily after birth. Rapid ductal growth and branching morphogenesis are induced during puberty, whereas the development of lobuloalveolar structures along the existing ductal tree takes place during pregnancy. At the end of gestation, alveolar epithelial cells are terminally differentiated, and acquire the ability to synthesize milk. The following milk secretion is triggered at parturition. After weaning, mammary glands undergo involution. During this period of time, secretory epithelial cells are apoptosed and stroma is remodeled to resume a quiescent state [Hennighausen and Robinson, 2001]. All of these features are delicately controlled by various hormones and growth factors.

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

W.-J. Wu, C.-F. Lee and C.-H. Hsin contributed equally to this study.

Grant sponsor: National Health Research Institute; Grant numbers: NHRI-EX93-9304SC, NHRI-EX94-9304SC; Grant sponsor: National Science Council; Grant number: NSC 92-2320-B-040-040; Grant sponsor: Chung Shan Medical University; Grant number: CSMU 89-OM-B-009.

*Correspondence to: Yi-Ju Lee, Institute of Immunology, Chung Shan Medical University, Taichung 402, Taiwan, ROC. E-mail: yijulee@csmu.edu.tw

Received 24 May 2007; Accepted 23 January 2008

DOI 10.1002/jcb.21734

Transforming growth factor-β (TGF)-β belongs to the TGF-β superfamily that regulates a wide spectrum of biological functions, such as proliferation, apoptosis, differentiation, and mobility. It also plays critical roles in development and carcinogenesis [Massague and Gomis, 2006]. Mammary gland development has been shown to be influenced by TGF-β at many stages. TGF-β inhibits ductal and branching morphogenesis during puberty, perturbs lobuloalveolar morphogenesis and differentiation during pregnancy, and promotes apoptosis during involution [Serra and Crowley, 2005]. Consistent with its global effects on mammary gland development, TGF-β is expressed throughout the whole period of postnatal development except during lactation [Robinson et al., 1991], implicating an undesired role of TGF-β in this stage of development. Indeed, evidence has revealed that transgenic mice expressing TGF-β1 or activated TGF-β type I receptor displayed a lactation-deficient phenotype with decreased lobuloalveolar density and increased apoptosis [Jhappan et al., 1993; Kordon et al., 1995; Siegel et al., 2003]. On the contrary, inhibition of TGF-β signaling by expressing truncated TGF-β type II receptor resulted in alveolar hyperplasia and precocious synthesis of a milk protein, β-casein, in virgin mice [Gorska et al., 1998]. Similar results were obtained by suppressing type II receptor expression via the antisense RNA strategy and gene knockout [Lenferink et al., 2003; Forrester et al., 2005]. These findings suggest that endogenous TGF-β prevents premature differentiation in mammary glands of virgin mice.

β-casein, a major component of milk proteins, is often used as a marker for mammary differentiation. Recapitulation of β-casein expression in vitro requires cell adhesion to basement membrane (BM) along with the stimulation of lactogenic hormones, which are prolactin, insulin, and hydrocortisone. This event is however inhibited by TGF-β [Robinson et al., 1993]. As reported earlier, TGF-β suppressed casein secretion but did not affect levels of casein mRNA [Robinson et al., 1993]. Results of pulse-chase labeling analysis further demonstrated that TGF-β-treated explants synthesized caseins at a lower rate than untreated, but the turnover of caseins was not altered [Robinson et al., 1993]. Given that the level of TGF-β in mammary glands elevates in mid-pregnancy, it has been proposed that TGF-β functions to limit the accumulation of milk caseins at this stage of development [Robinson et al., 1993]. Thus, the role of TGF-β here is fairly clear but the biochemical mechanism underlying it remains obscure.

TGF-β signals through a heterotetrameric receptor complex containing types I and II receptor serine/threonine kinases. The cascade of signaling is initiated when TGF-β binds to type II receptor, which then recruits, phosphorylates and activates type I receptor. Subsequently, R-Smads are phosphorylated by type I receptor, interact with Smad4, translocate into the nucleus and participate in transcription [Derynck and Zhang, 2003; Shi and Massague, 2003; Moustakas and Heldin, 2005]. In addition to the Smad pathway, numerous signaling molecules, including MAPK (Erk, Jnk, and p38), PI3K-Akt, Rho GTPase family, protein phosphatase 2A (PP2A) and PKC, are activated by TGF-β [Derynck and Zhang, 2003; Moustakas and Heldin, 2005]. Here we examined the pathways stimulated by TGF-β in primary mouse mammary epithelial cells and found that Smad3 was, at least in part, responsible for the inhibition of β-casein expression. Moreover, prolactin-induced tyrosine phosphorylation of Jak2 and Stat5 and serine/threonine phosphorylation of p70S6K and S6 ribosomal protein were suppressed by TGF-β. These might also have a role in downregulation of β-casein expression.

MATERIALS AND METHODS

Reagents

Bovine insulin, ovine prolactin, mouse EGF, and hydrocortisone were purchased from Sigma (St. Louis, MO). Recombinant human TGF-β1 was from R&D (Minneapolis, MN). Antibodies to phospho-Smad2, phospho-Erk, Erk, phospho-JNK, JNK, phospho-p38, p38, phospho-p70S6K, phospho-S6 ribosomal protein, phospho-eIF4E, phospho-4E-BP1 and phospho-Stat5 (Tyr694) were obtained from Cell Signaling (Beverly, MA). Antibodies to Stat5 and β-casein were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Jak2 and phosphotyrosine (4G10) were from Upstate Biotechnology (Lake Placid, NY). Antibody to Smad2/3 was from Becton Dickinson (Bedford, MA). ALK-5 inhibitor SB431542 was from Sigma. Kinase inhibitors SB203580 and PD98059 as well as the caspase inhibitor ZVAD-fmk were purchased from Calbiochem (San Diego, CA).

Cell Culture

Reconstituted BM matrix, Matrigel, was purchased from Becton Dickinson and coated onto dishes at 8 mg/ml. Primary epithelial cultures were prepared from mid-pregnant ICR mice and plated on Matrigel in nutrient mixture F-12 (Sigma) containing 10% fetal bovine serum (Hyclone), 1 mg/ml fetuin (Sigma), 5 ng/ml EGF, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. After 72 h, cells were serum-starved for 6–8 h in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12 (Invitrogen) containing hydrocortisone and insulin, and then subjected to various treatments. Mouse mammary gland epithelial cells, NMuMG, were obtained from Food Industry Research and Development Institute (Taiwan, ROC) and grown in DMEM supplemented with 10% fetal bovine serum and 10 μg/ml insulin.

Adenovirus Infection

Recombinant adenoviruses containing dominant negative Smad3, Smad3(D407E), and LacZ were kindly provided by Dr. Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). Adenoviral infection of primary mouse mammary epithelial cells was performed as previously described [Watkin and Streuli, 2002]. Briefly, mammary cells cultured on Matrigel were trypsinized and infected in suspension at 37°C for 1 h. Cells were then pelleted by centrifugation and plated onto Matrigel-coated dishes. After 24 h, medium was changed to DMEM/nutrient mixture F-12 containing hydrocortisone and insulin, and cells were stimulated with prolactin (3 μg/ml) or TGF-β1 (5 ng/ml).

Immunoprecipitation and Western Blot Analysis

Cells were lysed in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton-100. Normalization of protein was confirmed by SDS–PAGE, followed by Coomassie Blue staining. Cell lysates containing equal amounts of protein were incubated with 1–2 μg of antibody and 20–50 μl of protein A-Sepharose beads (Zymed Laboratories, Inc.) overnight at 4°C. Immunoprecipitates or whole cell lysates were subjected to SDS–PAGE, transferred to PVDF membrane (NEN), and probed with antibodies. Proteins were visualized using an ECL kit (Cell Signaling).

Reverse Transcription PCR (RT-PCR)

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. The first strand cDNA synthesis was carried out with ImProm-II™ reverse transcriptase (Promega) at 42°C for 1 h followed by incubation at 99°C for 5 min to denature reverse transcriptase. Amplification was performed in 15–22 cycles consisting of a denaturation step of 30 s at 94°C, an annealing step of 1 min at 61°C and an extension step of 90 s at 72°C. The PCR primers used for mouse β-casein were 5′-ATG CCC CTC CTT AAC TCT GAA-3′ (forward) and 5′-GCA TGA TCC AAA GGT GAA AAG-3′ (reverse); mouse GAPDH were 5′-ACC ACA GTC CAT GCC ATC AC-3′ (forward) and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (reverse); and mouse plasminogen activator inhibitor-1 (PAI-1) were 5′-TCA GAG CAA CAA GTT CAA CTA CAC TGA G-3′ (forward) and 5′- CCC ACT GTC AAG GCT CCA TCA CTT GCC CCA-3′ (reverse).

RESULTS

TGF-β Inhibits Prolactin-Induced Expression of β-Casein mRNA and Protein in Primary Mouse Mammary Epithelial Cells

We started with experiments to confirm the inhibitory effect of TGF-β on β-casein expression. Primary mammary epithelial cells derived from mammary glands of mid-pregnant mice were cultured on a reconstituted BM matrix, Matrigel, and exposed to varying concentrations of TGF-β and 3 μg/ml prolactin. Thirty six hours after stimulation, total cell lysates were collected and subjected to immunoblotting to measure the expression of β-casein protein. In agreement with the previous finding [Robinson et al., 1993], TGF-β suppressed prolactin-induced expression of β-casein protein in a dose-dependent manner (Fig. 1A). At the concentration of 5 ng/ml, TGF-β virtually abolished β-casein expression (Fig. 1A). This inhibition was achieved by concurrent application of both hormones to cells and was not further enhanced by prolonged treatment of TGF-β prior to prolactin (Fig. 1B).

We then inspected whether TGF-β inhibited β-casein expression at the mRNA level. As assessed by RT-PCR, the amount of β-casein

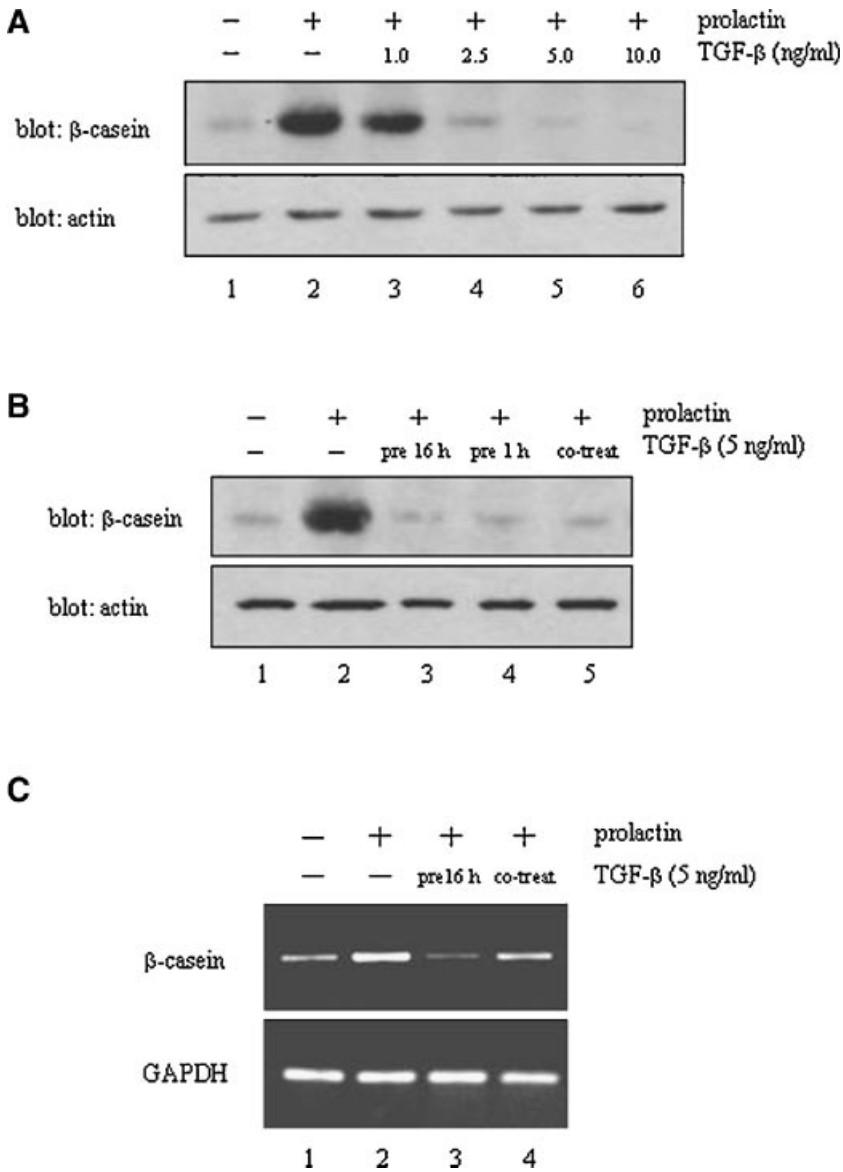

Fig. 1. TGF-β inhibits prolactin-induced expression of β-casein protein and mRNA. **A**: Primary mouse mammary epithelial cells cultured on BM were incubated with medium (**lane 1**), prolactin (3 μg/ml; **lane 2**), or simultaneously with prolactin and various concentrations of TGF-β (1–10 ng/ml) for 36 h (**lanes 3–6**). Total cell lysates were analyzed by immunoblotting with antibodies to β-casein and actin. To be noted that the TGF-β isoform used in this study was TGF-β1. **B**: Mammary cells cultured on BM were incubated with medium for 36 h (**lane 1**), prolactin for 36 h (**lane 2**), TGF-β (5 ng/ml) for 16 h before exposure to prolactin for 36 h (**lane 3**), TGF-β for 1 h before exposure to prolactin for 36 h (**lane 4**), or simultaneously with prolactin and TGF-β for 36 h (**lane 5**). Total cell lysates were analyzed by immunoblotting with antibodies to β-casein and actin. **C**: Mammary cells cultured on BM were incubated with medium for 24 h (**lane 1**), prolactin for 24 h (**lane 2**), TGF-β for 16 h before exposure to prolactin for 24 h (**lane 3**), or simultaneously with prolactin and TGF-β for 24 h (**lane 4**). Total RNA was reverse transcribed and PCR-amplified with primers for β-casein and GAPDH.

mRNA decreased by ~40% when cells were incubated with TGF-β and prolactin simultaneously for 24 h (Fig. 1C). Greater reduction occurred while TGF-β was applied to cells 16 h before prolactin (Fig. 1C). In order to find out whether TGF-β-mediated inhibition of β-casein mRNA expression was due to an increase in mRNA turnover, we included actinomycin D in cultures and assessed its effect on β-casein mRNA levels. Comparable results were obtained from cells treated with or without TGF-β (data not shown), suggesting that inhibition of β-casein mRNA expression by TGF-β occurred at the transcriptional level. Taken

together, our data here indicate that TGF-β downregulates prolactin-induced expression of β-casein mRNA and protein, although it seems to be more effective in the latter event.

Reduction of β-Casein Expression in Response to TGF-β Is Not Due to Cell Apoptosis

It has been shown that TGF-β induces apoptosis in a variety of cell types, including mammary epithelial cells. To clarify whether TGF-β-mediated inhibition of β-casein expression was secondary to its effect on cell death, we applied a general caspase inhibitor, ZVAD-fmk, along with TGF-β into cells to block apoptosis and examined if this would avoid downregulation of β-casein. Inclusion of ZVAD-fmk indeed decreased the amount of floating cells but did not alter the inhibitory effect of TGF-β on β-casein expression (Fig. 2). Therefore, lowering β-casein gene expression in response to TGF-β is not consequent on cell apoptosis. We speculate that other mechanisms are involved instead.

TGF-β Activates the Smad Pathway in Primary Mammary Epithelial Cells

To identify the signaling pathways responsible for the inhibitory effect of TGF-β, we would firstly find out the pathways activated by TGF-β in primary mammary epithelial cells. Numerous signaling routes have been shown to be triggered by TGF-β [Derynck and Zhang, 2003; Shi and Massague, 2003; Moustakas and Heldin, 2005]. Among them, the Smad and MAPK pathways are often reported. We thus focused on the examination of these pathways here. Phosphorylation of Smad2 was induced 5 min after TGF-β stimulation, peaked at 30 min and remained detectable up to 2 h (Fig. 2 and data not shown). There was no obvious induction of Erk, Jnk, and p38 phosphorylation within this time course (Fig. 3). These data thus suggest that TGF-β predominantly activates the Smad pathway in primary mouse mammary epithelial cells.

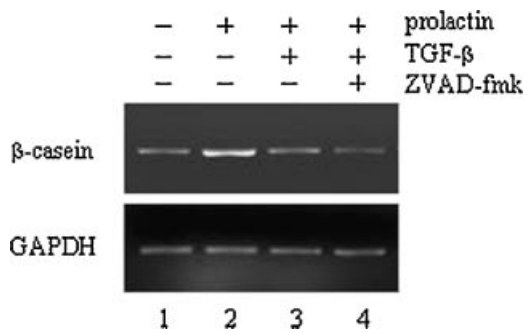

Fig. 2. Inclusion of caspase inhibitor ZVAD-fmk does not prevent TGF-β-mediated suppression of β-casein. Mammary cells cultured on BM were incubated with medium for 24 h (**lane 1**), prolactin for 24 h (**lane 2**), TGF-β for 16 h before exposure to prolactin for 24 h (**lane 3**), or simultaneously with TGF-β and ZVAD-fmk (100 μM) for 16 h before exposure to prolactin for 24 h (**lane 4**). Total RNA was reverse transcribed and PCR-amplified with primers for β-casein and GAPDH.

Inhibition of Type I TGF-β Receptor Kinase Activity by SB431542 Restores β-Casein Expression

We next examined whether the blockade of TGF-β-activated signaling pathways could restore prolactin-induced β-casein expression. SB431542, an inhibitor for ALK-5 (TGF-β receptor type I), did not affect prolactin-induced β-casein expression (Fig. 4A, lanes 2–5) but counteracted the inhibitory effect of TGF-β to fully resume β-casein expression (Fig. 4A, lanes 6–9). This was what we anticipated since ALK-5 acts fairly upstream of the TGF-β signaling cascade. By contrast, SB203580 and PD98059

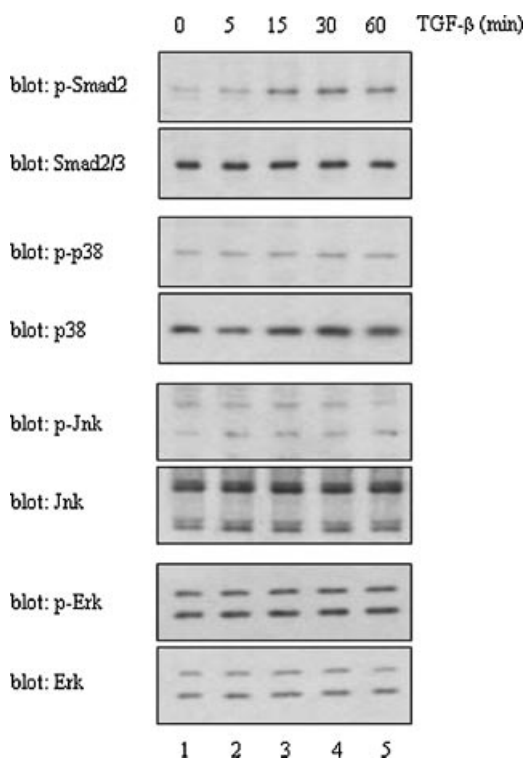

Fig. 3. Kinetic analysis of the signaling pathways activated by TGF-β. Mammary cells cultured on BM were incubated with TGF-β for 0–60 min. Total cell lysates were then analyzed by immunoblotting with the antibodies to phospho-Smad2, Smad2/3, phospho-p38, p38, phospho-Jnk, Jnk, phospho-Erk, and Erk.

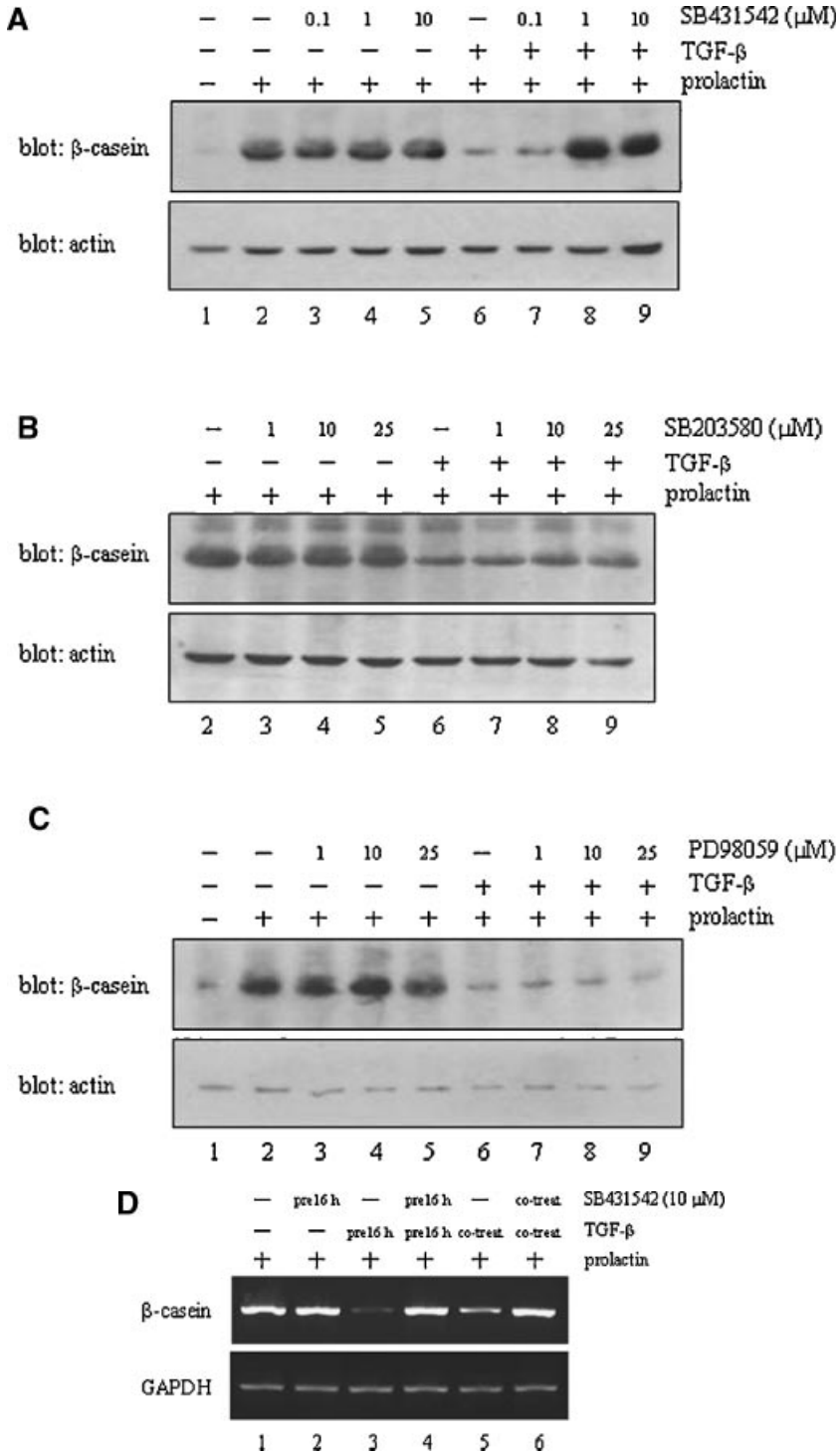

Fig. 4. Inhibition of type I TGF-β receptor kinase activity by SB431542 restores expression of β-casein mRNA and protein. **A–C**: Mammary cells cultured on BM were incubated with medium for 36 h (**lane 1**), prolactin for 36 h (**lane 2**), the indicated concentrations of kinase inhibitors for 1 h before exposure to prolactin for 36 h (**lanes 3–5**), TGF-β plus prolactin for 36 h (**lane 6**), or the indicated concentrations of kinase inhibitors for 1 h before exposure to both prolactin and TGF-β for 36 h (**lanes 7–9**). Kinase inhibitors used here were SB431542 (A), SB203580 (B), and PD98059 (C). Total cell lysates were analyzed by immunoblotting with antibodies to β-casein and actin. **D**: Mammary cells cultured on BM were incubated with prolactin for 24 h (**lane 1**), SB431542 (10 μM) for 16 h before exposure to prolactin for 24 h (**lane 2**), TGF-β for 16 h before exposure to prolactin for 24 h (**lane 3**), both SB431542 and TGF-β for 16 h before exposure to prolactin for 24 h (**lane 4**), TGF-β and prolactin for 24 h (**lane 5**), or simultaneously with SB431542, TGF-β, and prolactin for 24 h (**lane 6**). Total RNA was reverse transcribed and PCR-amplified with primers for β-casein and GAPDH.

which inhibit p38 and Mek, respectively, had no effect on β-casein expression whether cells were treated with prolactin or TGF-β (Fig. 4B,C). The p38 inhibitor SB203580 has been documented to inhibit types I and II TGF-β receptor activity [Eyers et al., 1998]; however, it did not negate the action of TGF-β in our experiment (Fig. 4B). We thus tested the ability of SB203580 to inhibit TGF-β-activated Smad2 phosphorylation in mammary epithelial cells. Our results revealed that SB203580 did not affect this signaling event (Supplemental Fig. 1). This is in agreement with other reports [Bakin et al., 2002; Yu et al., 2002], suggesting that the inhibitory effect of SB203580 on TGF-β receptor is cell type-dependent. Collectively, our data here were consistent with the aforementioned observation that neither p38 nor Erk was activated in mammary cells (Fig. 3). Thus, TGF-β-elicited inhibition of β-casein expression is not mediated by Erk or p38.

We also inspected the effect of SB431542 on β-casein mRNA expression. Just like it did for β-casein protein, SB431542 overrode the effect of TGF-β and completely restored prolactin-induced β-casein mRNA expression regardless of the timing that TGF-β was added (Fig. 4D). Therefore, SB431542 is effective in eradicating the detrimental effect of TGF-β on β-casein expression at both mRNA and protein levels.

Expression of Dominant Negative Smad3 Alleviates the Inhibitory Effect of TGF-β on β-Casein Expression

With the purpose of clarifying the involvement of the Smad pathway in the inhibitory effect of TGF-β on β-casein expression, a single missense mutant of Smad3, Smad3(D407E), was used. This mutant has been shown to be defective in phosphorylation in response to TGF-β [Goto et al., 1998]. Moreover, it blocks phoshorylation of both wild type Smad3 and Smad2, thereby exerting a dominant negative impact on TGF-β signaling [Goto et al., 1998]. To ensure its efficiency, we examined the effect of Smad3(D407E) on TGF-β-stimulated PAI-1 expression in a mouse mammary cell line NMuMG based on the fact that Smad3 is required for PAI-1 induction. As shown in Figure 5A, TGF-β stimulated the expression of PAI-1 mRNA, and this reaction was attenuated when cells were infected with adenoviral vector containing Smad3(D407E).

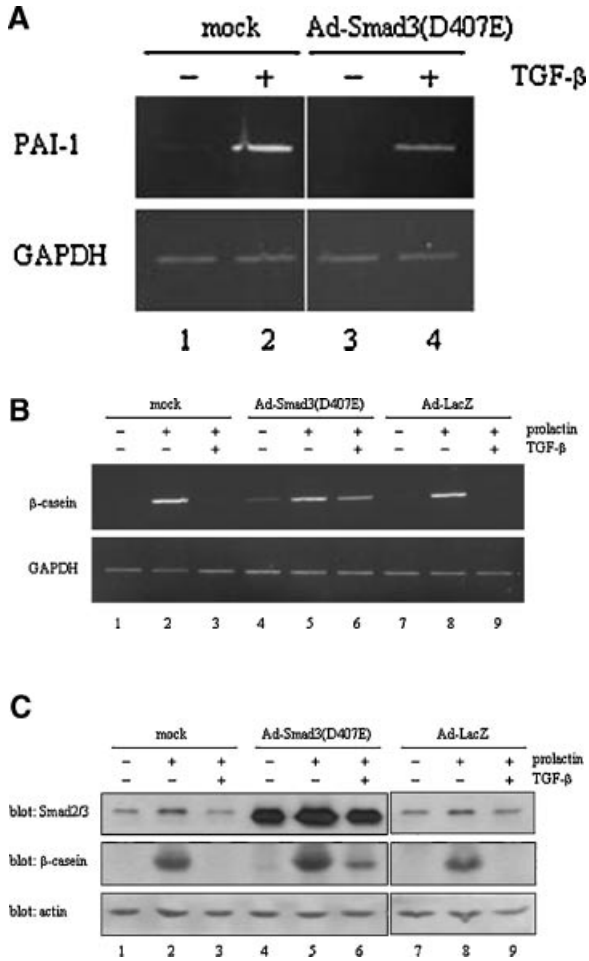

Fig. 5. Expression of dominant negative Smad3 alleviates the inhibitory effect of TGF-β on prolactin-induced β-casein expression. **A**: NMuMG cells were either mock-infected (**lanes 1–2**) or infected with recombinant adenovirus expressing dominant negative Smad3 [Ad-Smad3(D407E); **lanes 3–4**]. Twenty four hours after infection, cells were incubated in the absence of presence of TGF-β for 24 h. Total RNA was then reverse transcribed and PCR-amplified with primers for PAI-1 and GAPDH. **B**,**C**: Mammary cells infected with Ad-Smad3(D407E) (**lanes 4–6**), Ad-LacZ (**lanes 7–9**), or mock-infected (**lanes 1–3**) were incubated with medium (lanes 1, 4, and 7), prolactin (lanes 2, 5, and 8), or prolactin and TGF-β (lanes 3, 6, and 9) for 24 h. Total RNA was reverse transcribed and PCR-amplified with primers for β-casein and GAPDH (B), and cell lysates were analyzed by immunoblotting with antibodies to Smad2/3, β-casein, and actin (C).

After proving the efficiency of this mutant, we then tested its effect on restoring β-casein expression. Primary mammary cells were trypsinized, mock-infected or infected with adenoviral vector containing Smad3(D407E) (Ad-Smad3(D407E)) or LacZ (Ad-LacZ) in suspension for 1 h, seeded on Matrigel-coated dishes, cultured for 24 h and then stimulated

with prolatin in the absence or presence of TGF-β. Under the condition that prolactin and TGF-β were both added, cells overexpressing Smad3(D407E) exhibited higher extents of β-casein mRNA compared to mock-infected cells or Ad-LacZ-infected cells (Fig. 5B, lane 6 vs. lanes 3 and 9). Comparable results were also obtained for β-casein protein (Fig. 5C). Similar experiments were conducted using higher multiplicity of infection (MOI), and greater restoration of β-casein mRNA and protein expression was observed (Supplemental Fig. 2). Our data thus suggest that expression of dominant negative Smad3 mutant is able to alleviate the inhibitory effect of TGF-β. We also noticed a basal level of β-casein expression only in cells infected with Ad-Smad3(D407E) (Fig. 5B,C, lane 4), perhaps for the reason that the Smad3 mutant negated the action of traces of TGF-β in Matrigel. Moreover, different from the results in Figure 1C, cotreatment of TGF-β and prolactin here effectively blocked β-casein mRNA expression in these cells which were primary cells undergoing one round of trypsinization (Fig. 5B, lane 3). We are not certain what mechanism causes this difference but reckon that some factors derived from mouse mammary glands are sustained in primary cultures and function in protecting cells from the action of TGF-β. Trypsinization of cells might lead to the destabilization of these factors, leaving cells more susceptible to TGF-β's inhibition. Further experiments are needed to clarify this question. Collectively, our data here suggest that the Smad pathway is, at least in part, involved in TGF-β-mediated inhibition of β-casein expression.

Prolonged Treatment of TGF-β Leads to the Inhibition of Prolactin-Induced Tyrosine Phosphorylation of Jak2 and Stat5

In order to further delineate the mechanism for TGF-β-mediated inhibition of β-casein expression, we investigated into the possibility that TGF-β interfered with the Jak2-Stat5 pathway activated by prolactin. Mammary cells were pretreated with TGF-β for either 16 or 1 h before exposed to prolactin for 15 min, and cell lysates were then immunoprecipitated with antibodies to Jak2 and Stat5 followed by immunoblotting with anti-phosphotyrosine antibody. We found that prolonged incubation of TGF-β resulted in a decrease in levels of tyrosine phosphorylation of Jak2 and Stat5, whereas pretreatment of TGF-β for 1 h exerted no effect (Fig. 6). This was consistent with the result in Figure 1C that substantial down-regulation of β-casein mRNA took place only when cells were exposed to TGF-β long before prolactin.

Expression of Dominant Negative Smad3 Lessens the Inhibitory Effect of TGF-β on Prolatin-Induced Stat5 Tyrosine Phosphorylation

After discovering that TGF-β inhibited prolactin-stimulated signaling, we then further examined whether this was mediated by Smad3 as well. Mammary cells were mock-infected or infected with Ad-Smad3(D407E). After 24 h incubation, cells were pretreated with TGF-β for 16 h followed by stimulation with prolactin for 15 min. As shown in Figure 7, overexpression of Smad3(D407E) prevented TGF-β-induced massive inhibition of Stat5 tyrosine phosphorylation, and in these cells, Stat5 phosphorylation was elevated to a level ~45% of control (Fig. 7, compare lanes 4 to 3). Thus, TGF-β might hamper prolactin signaling by a mechanism involving Smad3.

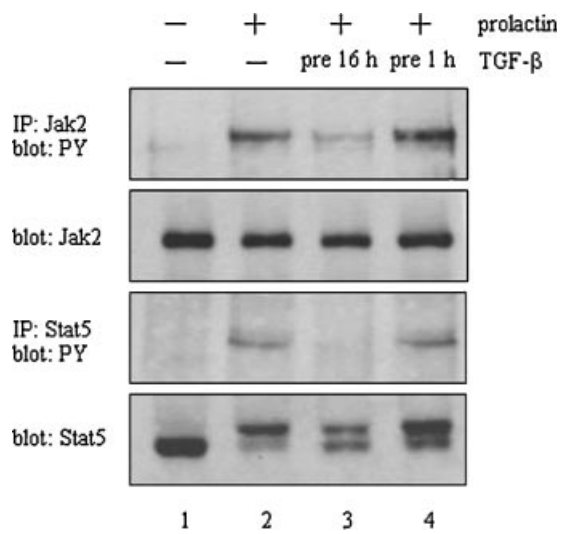

Fig. 6. Prolactin-induced tyrosine phosphorylation of Jak2 and Stat5 is inhibited by prolonged treatment of TGF-β. Mammary cells cultured on BM were incubated with medium for 15 min (**lane 1**), prolactin for 15 min (**lane 2**), or TGF-β for either 16 h (**lane 3**) or 1 h (**lane 4**) before exposure to prolactin for 15 min. Cell lysates were immunoprecipitated with antibody to Jak2 or Stat5 followed by immunoblotting with anti-phosphotyrosine antibody (PY). Expression levels of Jak2 and Stat5 were analyzed by immunoblotting with antibodies to Jak2 and Stat5.

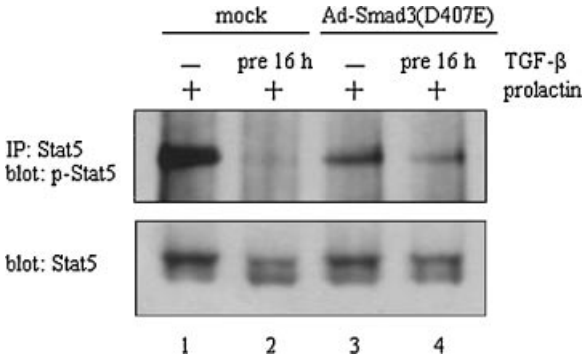

Fig. 7. Expression of dominant negative Smad3 lessens the inhibitory effect of TGF-β on prolactin-induced Stat5 tyrosine phosphorylation. Mammary cells infected with Ad-Smad3(D407E) (**lanes 3–4**) or mock-infected (**lanes 1–2**) were incubated with prolactin for 15 min (lanes 1 and 3) or pretreated with TGF-β for 16 h before exposure to prolactin for 15 min (lanes 2 and 4). Total cell lysates were immunoprecipitated with antibody to Stat5 followed by immunoblotting with anti-phospho-Stat5 (Tyr694) antibody. Expression level of Stat5 was analyzed by immunoblotting with antibody to Stat5.

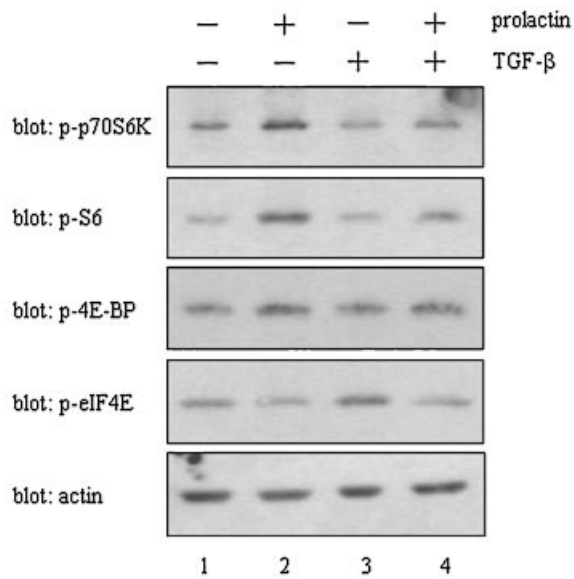

Fig. 8. TGF-β treatment decreases levels of phosphorylation of p70S6K and S6 ribosomal protein. Mammary cells cultured on BM were incubated with medium (**lane 1**), prolactin (**lane 2**), TGF-β (**lane 3**), or prolactin along with TGF-β (**lane 4**) for 8 h. Total cell lysates were then analyzed by immunoblotting with antibodies to phospho-p70S6K, phospho-S6 ribosomal protein, phospho-4E-BP, phospho-eIF4E, and actin.

TGF-β Inhibits Prolactin-Induced Serine/Threonine Phosphorylation of p70S6K and S6 Ribosomal Protein

One of the signaling molecules activated by TGF-β is PP2A. The Bα subunit of PP2A binds to type I TGF-β receptor in response to TGF-β; consequently, PP2A associates and inhibits p70S6K, leading to cell cycle arrest in G1 phase [Griswold-Prenner et al., 1998; Petritsch et al., 2000]. We were particularly interested in this pathway since p70S6K plays a role in regulation of translation. Besides, it has been shown that TGF-β-mediated inhibition of β-casein occurs at the translational level [Robinson et al., 1993]. Based on these facts, we examined the extents of phosphorylation of several proteins involved in translation, including p70S6K, S6 ribosomal protein, eIF-4E, and 4E-BP, under the condition that cells were incubated in the absence or presence of prolactin and TGF-β for 8 h. Prolactin stimulated phosphorylation of p70S6K and S6 ribosmal protein; whereas inclusion of TGF-β suppressed these responses by 43% and 53%, respectively ($P < 0.05$). A slight increase in levels of 4E-BP phosphorylation was observed when cells were stimulated with prolactin, but this was not altered by TGF-β. Likewise, no inhibition of eIF-4E phosphorylation by TGF-β was detected (Fig. 8). We also carried out similar experiments except for lengthening the incubation time to 24 h. Up to 75% and 77% of reduction in levels of phosphorylation of p70S6K and S6 ribosomal protein, respectively, was observed in response to TGF-β ($P < 0.05$; Supplemental Fig. 3). Thus, TGF-β perturbs prolactin-induced phosphorylation of p70S6K and S6 ribosomal protein, which might subsequently cause the ineffectiveness in β-casein protein synthesis.

DISCUSSION

The key development of mammary gland occurs post-natally. Upon each pregnancy, mammary gland undergoes a course of proliferation, differentiation and involution, accompanied by dramatic changes in morphology. Regulation of this process requires intricate interplay of multiple growth factors, hormones and extracellular matrix. TGF-β is expressed at all phases of mammary gland development except during lactation, stressing its adverse effect on mammary function at this period of time. In fact, TGF-β has been documented to inhibit the synthesis of milk protein genes, such as β-casein. Here we further demonstrate that this inhibitory effect is mediated by a Smad3-dependent mechanism. Moreover, prolactin-induced tyrosine phosphorylation of Jak2

and Stat5 and serine/threonine phosphorylation of p70S6K and S6 ribosomal protein are suppressed by TGF-β. These might contribute to the downregulation of transcription and translation of the β-casein gene.

In this study, we found that TGF-β inhibited β-casein expression at both mRNA and protein levels (Fig. 1). This is different from an observation reported earlier in that only β-casein protein is repressed [Robinson et al., 1993]. One possible explanation for this discrepancy is that the concentration of prolactin (5 μg/ml) and glucocorticoid (5 μg/ml) they used is higher than those we did (3 μg/ml prolactin and 1 μg/ml glucocorticoid); furthermore, these hormones have been shown to hinder TGF-β response [Woo et al., 1996; Song et al., 1999; Bailey et al., 2004]. Prolactin impeded TGF-β-induced apoptosis in an Akt-dependent fashion in mammary epithelial cells [Bailey et al., 2004]. The role of Akt in counteracting TGF-β signaling was then characterized in other studies, illustrating that Akt interacted with Smad3 and blocked Smad3 phosphorylation, association with Smad4 and the subsequent translocation into the nucleus [Conery et al., 2004; Remy et al., 2004]. Like prolactin, glucocorticoid also influences TGF-β responses albeit both positive and negative effects have been reported. In mammary cells, glucocorticoid and TGF-β act against each other to regulate tight junction dynamics [Woo et al., 1996]. This antagonism can be partly explained by a later finding that glucocorticoid receptor binds Smad3 and inhibits Smad3-mediated transcription [Song et al., 1999]. Accordingly, higher dose of prolactin and glucocorticoid would results in greater activation of Akt and glucocorticoid receptor, which then negate the act of Smad3 by physical sequestration or functional inhibition of Smad3. Another explanation for the difference in results regarding the effect of TGF-β on β-casein mRNA expression is the duration of hormone stimulation, which is 48 and 24 h in their and our studies, respectively. It has been shown that prolonged treatment of lactogenic hormones greatly enhances β-casein mRNA stability [Poyet et al., 1989]. Therefore, massive accumulation of β-casein mRNA under this condition would mask the diminution derived from TGF-β-mediated inhibition of transcription.

Multiple transcription factors are involved in transcription of β-casein gene, including Stat5, glucocorticoid receptor, C/EBPβ, NF-1, Oct-1 and Runx2/cbfa1. Conceivably, suppression of the expression or function of these factors would lead to a decrease in β-casein mRNA expression. Here we found that prolonged treatment of TGF-β reduced levels of tyrosine phosphorylation of Jak2 and Stat5 (Fig. 6), which might thus have a part in attenuation of β-casein mRNA expression. Similar results have been detected in other studies, and the possible mechanisms for it were revealed recently [Bright et al., 1997; Fox et al., 2003; Lovibond et al., 2003; Roes et al., 2003; Park et al., 2005]. One involves suppressor of cytokine stimulation 3 (SOCS3), a negative regulator of the Jak/Stat pathway. SOCS3 is induced by TGF-β in bone marrow precursor cells and this induction facilitates osteoclast lineage commitment by blocking the inhibitory effect of IFN-β [Fox et al., 2003; Lovibond et al., 2003]. The other mechanism involves the protein tyrosine phosphatase (PTP), SHP-1. TGF-β stimulates the expression of SHP-1 in $CD4^+$ T cells, which then play a role in perturbing IFN-γ-induced Jak/Stat signaling [Park et al., 2005]. Interestingly, these aforementioned TGF-β responses, including the induction of SOCS and PTP, are all mediated through upregulation of gene expression, correlating with our result that disruption of the Jak/Stat pathway by TGF-β requires prolonged treatment of TGF-β (Fig. 6). As we found that Smad3 contributed to TGF-β-mediated inhibition of Stat5 tyrosine phosphorylation (Fig. 7), it is appealing to propose that Smad3-mediated transcription accounts for induction of SOCS and PTP, thereby conferring the inhibitory effect of TGF-β. Further investigation is required to address this question.

It is likely that TGF-β inhibits β-casein mRNA expression by interfering the expression or function of transcription factors other than Stat5. One candidate is C/EBPβ, an indispensable transcription factor for β-casein gene. Mounting data have shown that TGF-β suppresses C/EBP-mediated transcription by inhibiting the expression, DNA-binding or transactivation of C/EBP, and the signaling molecule conferring these actions is Smad3 [Choy and Derynck, 2003; Feinberg et al., 2004; Ahdjoudj et al., 2005]. The other potential target of TGF-β's inhibitory effect is Runx2/cbfa1. Runx2/cbfa1 is known for its role in osteoblast differentiation, but TGF-β inhibits this process by repressing the expression and transactivation of Runx2/cbfa1 [Alliston et al.,

2001]. Again, Smad3 was verified to mediate this effect. It either promotes the recruitment of histone deacetylase to Runx2/cbfa1 at the Runx2/cbfa1-binding site [Kang et al., 2005], or augments the expression of Msx2, an antagonist of Runx2/cbfa1, to control Runx2/cbfa1 activity [Hjelmeland et al., 2005]. However, the possibility that TGF-β thwarts the function of glucocorticoid receptor, another important transcription factor for β-casein transcription, is probably minute because TGF-β has been shown to elicit no effect on glucocorticoid receptor-driven transcription [Song et al., 1999].

The earlier study from Robinson et al. [1993] suggests that TGF-β inhibits β-casein expression at the translational level. This is, to some extent, in agreement with our results: (1) TGF-β is more effective in blocking β-casein protein expression than in blocking mRNA expression (Fig. 1); and (2) TGF-β decreases levels of prolactin-induced serine/threonine phosphorylation of p70S6K and S6 ribosomal protein (Fig. 8). S6K and its upstream activator, the mammalian target of rapamycin (mTOR), are crucial in the regulation of translation. In response to stimulation, mTOR is recruited to the eIF3 complex where S6K has already bound, and phosphorylates S6K [Holz et al., 2005]. This facilitates S6K dissociation from the eIF3 complex and the subsequent full activation. Consequently, S6K phosphorylates eIF2B and the 40S ribosomal protein S6 [Holz et al., 2005]. Active mTOR also phosphorylates 4E-BP, leading to the release of 4E-BP from eIF4E; in turn, eIF4G is recruited and bridges between eIF4E at 5′-cap and polyA binding protein (PABP) at 3′-end of mRNA. In contrast to mTOR and S6K, S6 ribosomal protein's role in translation is rather ambiguous. S6 phosphorylation was initially thought to promote the translation of mRNA containing 5′-terminal oligopyrimidine tract [Jefferies et al., 1994], but this concept was recently challenged by the observation that cells defective in S6 phosphorylation were still competent in translation of this set of mRNA [Ruvinsky et al., 2005]. On the contrary, a positive role of S6 phosphorylation in cap-dependent translation has just been reported [Roux et al., 2007]. It shows that S6 interacts with the 7-methylguanosine cap complex when it is phosphorylated at Ser235/236 [Roux et al., 2007]. Collectively, the mTOR/S6K signaling cascade and possibly S6 phosphorylation are involved in the regulation of translation initiation. In this regard, downregulation of these events, as we observed in this study (Fig. 8), would have a negative impact on protein synthesis. This notion is somewhat supported by an earlier finding that application of rapamycin into cultures of mouse mammary explants inhibits prolactin-induced casein synthesis [Hang and Rillema, 1997].

It is currently unclear how TGF-β suppresses serine/threonine phosphorylation of p70S6K and S6 ribosomal protein in our system. We speculate that PP2 mediates this effect because TGF-β has been shown to inhibit p70S6K via PP2A to induce cell cycle arrest in a mammary epithelial cell line, Eph4 [Petritsch et al., 2000]. The regulatory Bα subunit of PP2A, responsible for interacting with type I TGF-β receptor, is a WD40 repeat-containing protein [Petritsch et al., 2000]. To be noted that several WD40 repeat proteins are bound to TGF-β receptors as well [Derynck and Zhang, 2003]. Among them, eIF2α and TGF-β-receptor-interacting protein 1 (TRIP-1) which is a subunit of the eIF3 complex are particularly of interest to us since both them are part of the translation initiation complex. However, there is no evidence that TGF-β influences translation via TRIP-1 and eIF2α, and so far their role in TGF-β response has been confined to a modulator of TGF-β receptor signaling.

Synthesis of milk to nourish the offspring is the ultimate function of mammary glands. A number of hormones and growth factors orchestrate this process, and the intricacy of it is represented in the regulation β-casein. Induction of β-casein by lactogenic hormones is modulated at transcriptional, post-transcriptional and translational levels, whereas inhibition of it by TGF-β is perhaps through both transcriptional and translational controls although the latter seems to be more important. Based on our data and other observations in related reports, we reckon that short exposure to TGF-β could cause some extents of inhibition of β-casein mRNA expression, possibly through the negative effect of Smad3 on C/EBP or cbfa-1/Runx2; but effective downregulation of β-casein mRNA is achieved by further impairing the Jak2/Stat5 pathway, and this probably involves the induction of other genes such as PTP or SOCS. On the other hand, abrogation of β-casein protein expression results partly from a decrease in mRNA expression which is

Smad3-dependent, and from the reduction of translation efficiency mediated by perturbation of phosphorylation of p70S6K and S6 ribosomal protein. The latter event is, as we speculate, ascribed to TGF-β-activated PP2 activity.

ACKNOWLEDGMENTS

We thank Dr. Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden) for kindly providing adenoviral constructs, AdX-Smad3(D407E) and AdX-LacZ, Dr. Gwo-Tarng Sheu for critical reading of the manuscript and Dr. Jiun-Liang Ko for helpful suggestions.

REFERENCES

Ahdjoudj S, Kaabeche K, Holy X, Fromigue O, Modrowski D, Zerath E, Marie PJ. 2005. Transforming growth factor-beta inhibits CCAAT/enhancer-binding protein expression and PPARgamma activity in unloaded bone marrow stromal cells. Exp Cell Res 303:138–147.

Alliston T, Choy L, Ducy P, Karsenty G, Derynck R. 2001. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. EMBO J 20:2254–2272.

Bailey JP, Nieport KM, Herbst MP, Srivastava S, Serra RA, Horseman ND. 2004. Prolactin and transforming growth factor-beta signaling exert opposing effects on mammary gland morphogenesis, involution, and the Akt-forkhead pathway. Mol Endocrinol 18:1171–1184.

Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. 2002. p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration. J Cell Sci 115:3193–3206.

Bright JJ, Kerr LD, Sriram S. 1997. TGF-beta inhibits IL-2-induced tyrosine phosphorylation and activation of Jak-1 and Stat 5 in T lymphocytes. J Immunol 159:175–183.

Choy L, Derynck R. 2003. Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. J Biol Chem 278:9609–9619.

Conery AR, Cao Y, Thompson EA, Townsend CM, Jr., Ko TC, Luo K. 2004. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. Nat Cell Biol 6:366–372.

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425:577–584.

Eyers PA, Craxton M, Morrice N, Cohen P, Goedert M. 1998. Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution. Chem Biol 5:321–328.

Feinberg MW, Watanabe M, Lebedeva MA, Depina AS, Hanai J, Mammoto T, Frederick JP, Wang XF, Sukhatme VP, Jain MK. 2004. Transforming growth factor-beta1 inhibition of vascular smooth muscle cell activation is mediated via Smad3. J Biol Chem 279:16388–16393.

Forrester E, Chytil A, Bierie B, Aakre M, Gorska AE, Sharif-Afshar AR, Muller WJ, Moses HL. 2005. Effect of conditional knockout of the type II TGF-beta receptor gene in mammary epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. Cancer Res 65:2296–2302.

Fox SW, Haque SJ, Lovibond AC, Chambers TJ. 2003. The possible role of TGF-beta-induced suppressors of cytokine signaling expression in osteoclast/macrophage lineage commitment in vitro. J Immunol 170:3679–3687.

Gorska AE, Joseph H, Derynck R, Moses HL, Serra R. 1998. Dominant-negative interference of the transforming growth factor beta type II receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. Cell Growth Differ 9:229–238.

Goto D, Yagi K, Inoue H, Iwamoto I, Kawabata M, Miyazono K, Kato M. 1998. A single missense mutant of Smad3 inhibits activation of both Smad2 and Smad3, and has a dominant negative effect on TGF-beta signals. FEBS Lett 430:201–204.

Griswold-Prenner I, Kamibayashi C, Maruoka EM, Mumby MC, Derynck R. 1998. Physical and functional interactions between type I transforming growth factor beta receptors and Balpha, a WD-40 repeat subunit of phosphatase 2A. Mol Cell Biol 18:6595–6604.

Hang J, Rillema JA. 1997. Effect of rapamycin on prolactin-stimulated S6 kinase activity and milk product formation in mouse mammary explants. Biochim Biophys Acta 1358:209–214.

Hennighausen L, Robinson GW. 2001. Signaling pathways in mammary gland development. Dev Cell 1:467–475.

Hjelmeland AB, Schilling SH, Guo X, Quarles D, Wang XF. 2005. Loss of Smad3-mediated negative regulation of Runx2 activity leads to an alteration in cell fate determination. Mol Cell Biol 25:9460–9468.

Holz MK, Ballif BA, Gygi SP, Blenis J. 2005. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell 123:569–580.

Jefferies HB, Reinhard C, Kozma SC, Thomas G. 1994. Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. Proc Natl Acad Sci USA 91:4441–4445.

Jhappan C, Geiser AG, Kordon EC, Bagheri D, Hennighausen L, Roberts AB, Smith GH, Merlino G. 1993. Targeting expression of a transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. EMBO J 12:1835–1845.

Kang JS, Alliston T, Delston R, Derynck R. 2005. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. EMBO J 24:2543–2555.

Kordon EC, McKnight RA, Jhappan C, Hennighausen L, Merlino G, Smith GH. 1995. Ectopic TGF beta 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. Dev Biol 168:47–61.

Lenferink AE, Magoon J, Pepin MC, Guimond A, O'Connor-McCourt MD. 2003. Expression of TGF-beta type II receptor antisense RNA impairs TGF-beta signaling in vitro and promotes mammary gland differentiation in vivo. Int J Cancer 107:919–928.

Lovibond AC, Haque SJ, Chambers TJ, Fox SW. 2003. TGF-beta-induced SOCS3 expression augments TNF-alpha-induced osteoclast formation. Biochem Biophys Res Commun 309:762–767.

Massague J, Gomis RR. 2006. The logic of TGFbeta signaling. FEBS Lett 580:2811–2820.

Moustakas A, Heldin CH. 2005. Non-Smad TGF-beta signals. J Cell Sci 118:3573–3584.

Park IK, Shultz LD, Letterio JJ, Gorham JD. 2005. TGF-beta1 inhibits T-bet induction by IFN-gamma in murine CD4+ T cells through the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1. J Immunol 175:5666–5674.

Petritsch C, Beug H, Balmain A, Oft M. 2000. TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. Genes Dev 14:3093–3101.

Poyet P, Henning SJ, Rosen JM. 1989. Hormone-dependent beta-casein mRNA stabilization requires ongoing protein synthesis. Mol Endocrinol 3:1961–1968.

Remy I, Montmarquette A, Michnick SW. 2004. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. Nat Cell Biol 6:358–365.

Robinson SD, Silberstein GB, Roberts AB, Flanders KC, Daniel CW. 1991. Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. Development 113:867–878.

Robinson SD, Roberts AB, Daniel CW. 1993. TGF beta suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy. J Cell Biol 120:245–251.

Roes J, Choi BK, Cazac BB. 2003. Redirection of B cell responsiveness by transforming growth factor beta receptor. Proc Natl Acad Sci USA 100:7241–7246.

Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, Sonenberg N, Blenis J. 2007. RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. J Biol Chem 282:14056–14064.

Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zisman P, Meyuhas O. 2005. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes Dev 19:2199–2211.

Serra R, Crowley MR. 2005. Mouse models of transforming growth factor beta impact in breast development and cancer. Endocr Relat Cancer 12:749–760.

Shi Y, Massague J. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685–700.

Siegel PM, Shu W, Cardiff RD, Muller WJ, Massague J. 2003. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. Proc Natl Acad Sci USA 100: 8430–8435.

Song CZ, Tian X, Gelehrter TD. 1999. Glucocorticoid receptor inhibits transforming growth factor-beta signaling by directly targeting the transcriptional activation function of Smad3. Proc Natl Acad Sci USA 96:11776–11781.

Watkin H, Streuli CH. 2002. Adenoviral-mediated gene transfer in two-dimensional and three-dimensional cultures of mammary epithelial cells. Methods Cell Biol 69: 403–423.

Woo PL, Cha HH, Singer KL, Firestone GL. 1996. Antagonistic regulation of tight junction dynamics by glucocorticoids and transforming growth factor-beta in mouse mammary epithelial cells. J Biol Chem 271:404–412.

Yu L, Hebert MC, Zhang YE. 2002. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J 21:3749–3759.