

Serotonin Suppresses β -Casein Expression *via* Inhibition of the Signal Transducer and Activator of Transcription 5 (STAT5) Protein Phosphorylation in Human Mammary Epithelial Cells MCF-12A

Takeshi Chiba,^{*,a} Soichiro Kimura,^b Katsuo Takahashi,^a Yasunori Morimoto,^b Atsushi Sanbe,^c Hideo Ueda,^b and Kenzo Kudo^a

^aDepartment of Clinical Pharmaceutics and Pharmacy Practice, School of Pharmacy, Iwate Medical University;

^cDepartment of Pharmacotherapeutics, School of Pharmacy, Iwate Medical University; 2-1-1 Nishitokuta, Yahaba-cho, Shiwa-gun, Iwate 028-3603, Japan: and ^bDepartment of Hospital Pharmacy, Faculty of Pharmaceutical Sciences, Josai University; 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan.

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Serotonin (5-hydroxytryptamine; 5-HT) has an important physiological role in controlling lactation, namely, milk volume homeostasis, within mammary glands. The objectives of this study were to evaluate whether exogenous 5-HT can suppress β -casein expression, a differentiation marker, produced in human mammary epithelial cells, and to determine whether 5-HT can attenuate β -casein signaling *via* the prolactin (PRL) receptor (PRLr) and Janus kinase 2/signal transducer and activator of transcription 5 (STAT5) pathway. PRL treatment increased the mRNA level of β -casein in the MCF-12A human mammary epithelial cell line, and the highest level occurred at days 7 and 14 of culture. In contrast, PRLr expression was not affected significantly by PRL treatment. PRL treatment in MCF-12A cells increased levels of β -casein and phosphorylated STAT5 (pSTAT5) proteins in a concentration-dependent manner, with a slight increase of STAT5 protein. β -Casein expression was inhibited by 0.1 mM 5-HT in a time-dependent manner. Additionally, treatment with 0.1 mM 5-HT for 72 h decreased protein levels of β -casein and pSTAT5, with a slight decrease in STAT5 levels. These results suggest that exogenous 5-HT can inhibit STAT5 phosphorylation, resulting in a decrease in β -Casein expression. In conclusion, we showed that exogenous 5-HT decreased β -casein expression in MCF-12A human mammary epithelial cells, and that 5-HT was responsible for inhibiting phosphorylation of STAT5, resulting in a decline in lactational function.

Key words serotonin; β -casein; human mammary gland; MCF-12A; milk protein

The mammary gland, known as an exocrine gland, in female mammals is responsible for milk production as the only food source that provides nutrition to infants.¹⁾ The characteristics of the adult mammary gland are altered by numerous stimuli during pregnancy, and then the glands gain the function of milk production during lactation. The development and maturation of mammary epithelium are required in the mammary gland, and these processes are regulated by a dynamic interaction between endocrine hormones and locally produced factors. Lack of demand for milk at weaning culminates milk production and initiates the process of involution, whereby the mammary epithelium is remodeled back to its pre-pregnancy state.^{2,3)}

Prolactin (PRL), an anterior pituitary hormone, is essential for mammary epithelium development and milk production. PRL binds to a PRL receptor (PRLr) on the epithelial cell membrane, resulting in activation of several signaling pathways, including the Janus kinase 2/signal transducer and activator of transcription 5 (Jak2/STAT5), mitogen-activated protein kinase, and phosphoinositide 3-kinase pathways.⁴⁻⁶⁾ Particularly, signaling *via* the Jak2/STAT5 pathway can promote the expression of genes encoding milk proteins such as β -casein, whose expression is an important differentiation marker in mammary epithelial cells, and whey acidic protein, which is associated with the PRL-phosphorylated STAT5 (pSTAT5) pathway.⁷⁻¹¹⁾

In addition to PRL, serotonin (5-hydroxytryptamine; 5-HT) is also associated with milk production in mammary gland

epithelial cells. 5-HT is classically known as a neurotransmitter involved in various physiological states and behaviors, such as regulating sleep, appetite, memory, sexual behavior, neuroendocrine function, and mood.¹²⁾ 5-HT has an important physiological role in controlling milk volume homeostasis within mammary glands. When the mammary gland is filled with milk during lactation, 5-HT provides negative feedback signals to suppress milk synthesis.^{13,14)} In addition, continuation of ab lactation accelerates 5-HT action, triggering the beginning stage of involution.¹⁴⁾

Recent studies showed that accelerated 5-HT action increases permeability of tight junctions *via* a 5-HT₇ receptor, which is an important event initiating involution, and suppresses β -casein mRNA in primary mouse and bovine mammary epithelial cells.^{14,15)} In addition, fluoxetine, which is a selective serotonin reuptake inhibitor (SSRI), and increased levels of extracellular 5-HT also inhibit milk protein genes such as β -casein and α -lactalbumin in primary bovine mammary epithelial cells.¹⁶⁾ However, there have been no studies examining whether 5-HT inhibits expression of genes encoding milk proteins such as β -casein in human mammary epithelial cells.

In the present study, MCF-12A human mammary epithelial cells were examined to evaluate whether PRL treatment increases β -casein expression and whether its increase is attributed to activation of the Jak2/STAT5 signaling pathway. Subsequently, we also determined the effect of 5-HT treatment on β -casein protein produced by MCF-12A cells and on the Jak2/STAT5 signaling pathway.

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: chibat@iwate-med.ac.jp

MATERIALS AND METHODS

Cell Culture MCF-12A cells were obtained from the American Type Culture Collection (VA, U.S.A.) and were used at passages 58–62. Cells were cultured in a growth medium (GM) of Dulbecco's modified Eagle's medium (DMEM):F12 (1:1; Invitrogen, CA, U.S.A.) supplemented with 10 μ g/mL human insulin (Sigma-Aldrich, MO, U.S.A.), 0.5 μ g/mL hydrocortisone (Sigma-Aldrich), 20 ng/mL human recombinant epithelial growth factor (hEGF; BD Biosciences, MA, U.S.A.), 5% horse serum (Invitrogen), 100 IU/mL penicillin, and 100 μ g/mL streptomycin (Sigma-Aldrich). The cells were seeded at a density of 1×10^5 cells/cm² on dishes or plates coated with growth factor reduced Matrigel® (BD Biosciences) and maintained under an atmosphere of 95% air and 5% CO₂ at 37°C. Unless otherwise stated, the GM was changed to a differentiation medium (DM) modified by addition of PRL (0.01 μ g/mL or 0.1 μ g/mL) and removal of hEGF, 24 h after cell seeding. The DM was changed every 2 d. For cells treated with 5-HT (Wako Pure Chemical Industries, Ltd., Osaka, Japan), no horse serum was added to the DM, and 5-HT was added to the DM at day 7 of culture.

Cell Viability An assay was conducted to estimate cell viability using a Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan). Briefly, MCF-12A cells were seeded onto 96-well plates at a density of 1×10^5 cells/cm². At each time point, the supernatant was removed, and 100 μ L of GM and 10 μ L of CCK8 were added to each well. After incubation for 2 h at 37°C, the absorbance was recorded at 450 nm using a microplate reader (SH-1200Lab; HITACHI, Tokyo, Japan).

Quantitative Real-Time (RT)-Polymerase Chain Reaction (PCR) Total RNA was isolated from the cultured cells using an RNeasy Mini Kit and DNase Set (Qiagen, CA, U.S.A.). cDNA was prepared using a High-Capacity RNA to cDNA™ Kit (Applied Biosystems, CA, U.S.A.). The mRNA levels of related genes were evaluated using a 7500 Real-Time PCR System (Applied Biosystems) with TaqMan Universal Master Mix II (Applied Biosystems), under the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 60 cycles of 95°C for 15 s and 60°C for 1 min. The pre-designed primer and probe sets for human β -casein, *PRLr*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were commercially available (Applied Biosystems; β -casein, Hs_00914395_m1; *PRLr*, Hs_00168739_m1; *GAPDH*, Hs_02758991_g1). Quantitative values were obtained from the threshold cycle number. The mRNA level of the target gene was normalized to the *GAPDH* mRNA content for each sample. All samples were analyzed in triplicate, and average quantities of the gene transcripts were used for calculation.

Western Blotting Protein was extracted from cultured cells using M-PER® Mammalian Protein Extraction Reagent (Pierce, IL, U.S.A.), and its concentration was determined by a BCA™ Protein Assay Kit (Pierce). Proteins were then subjected to electrophoresis on 7.5% or 12.5% e-PAGE (ATTO, Tokyo, Japan) then transferred onto polyvinylidene difluoride membranes (GE Healthcare, Tokyo, Japan). The membranes were probed overnight at 4°C with primary antibodies specific for β -casein (1:400; Novus Biologicals, CO, U.S.A.), STAT5 (1:1000; Abcam, Tokyo, Japan), pSTAT5 (1:200; Abcam), or *GAPDH* (1:2000; Calbiochem, CA, U.S.A.). Proteins were visualized with horseradish-peroxidase-conjugated secondary

antibodies (1:2000; Santa Cruz, TX, U.S.A.) and observed using an Image Reader LAS-3000 System (FUJIFILM, Tokyo, Japan). Immunoblot films were scanned, and blot density was analyzed using Image J ver.1.47 software.

Statistical Analysis All reported values were expressed as mean \pm standard error of the mean (S.E.M.). In comparison between multiple groups, data were analyzed by one-way ANOVA followed by the Dunnett's test to compare each group with the control. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Influence of PRL Treatment on the mRNA Levels of β -Casein and *PRLr* in MCF-12A Cells MCF-12A cells were cultured for 24 h in GM after seeding, and then the GM was replaced with DM containing 0.1 μ g/mL of PRL. The cells maintained viability until day 14 of culture (Fig. 1). Figure 2

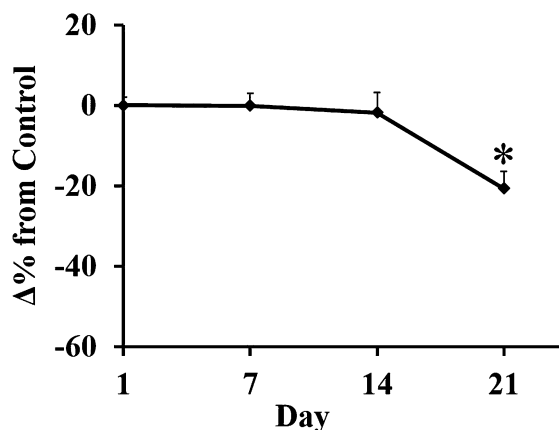


Fig. 1. Viability Curve for MCF-12A Cells Treated with PRL

The cells were grown in GM for 1 d after seeding, and then the GM was replaced with DM containing 0.1 μ g/mL PRL. Cell viability was measured by Cell Counting Kit-8 at days 1, 7, 14, and 21 of culture. Data were expressed as percent difference of the cells cultured for 1 d. The values are the mean \pm S.E.M. ($n=3-4$). Asterisks (*) indicate significantly different results (Dunnett's test, $p < 0.05$, versus value at day 1).

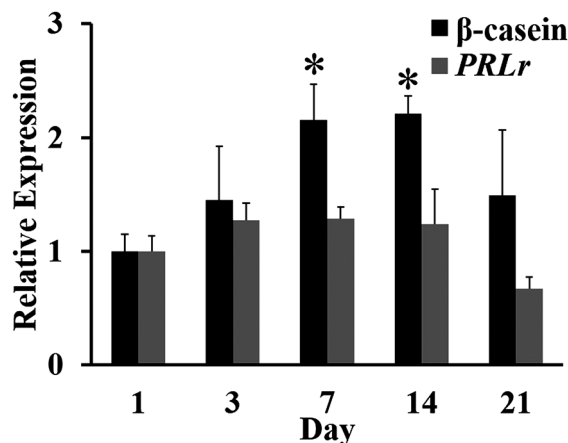


Fig. 2. Effect of PRL Treatment on mRNA Expression of β -Casein and *PRLr* in MCF-12A Cells

The relative β -casein and *PRLr* mRNA levels were analyzed by quantitative RT-PCR in the cells cultured for varying periods, compared to levels of the internal standard *GAPDH*. Closed and shaded bars indicate the relative expression of β -casein and *PRLr*, respectively. The values are the means \pm S.E.M. ($n=3-4$). Asterisks (*) indicate significantly different results (Dunnett's test, $p < 0.05$, compared with day 1).

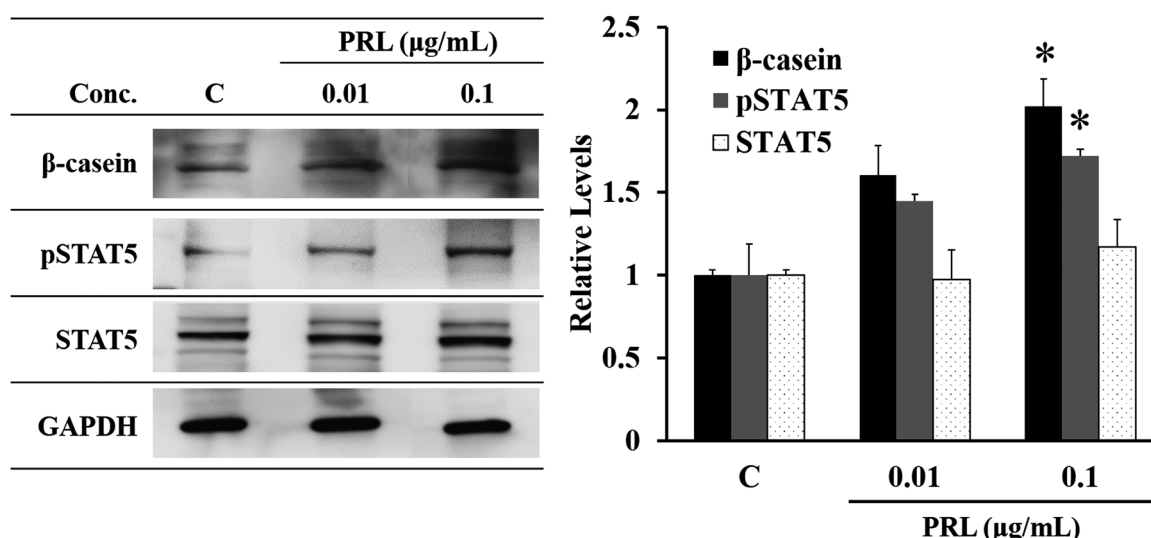


Fig. 3. Effects of PRL Treatment on Protein Levels of β -Casein, pSTAT5, and STAT5 in MCF-12A Cells

The relative protein levels of β -casein, pSTAT5, and STAT5 in PRL-treated (PRL) and untreated cells (C) were analyzed by Western blot, compared to that of the internal standard GAPDH. Closed, shaded, and dotted bars indicate the relative levels of β -casein, pSTAT5, and STAT5, respectively. The values are the means \pm S.E.M. ($n=3-4$). Asterisks (*) indicate significantly different results (Dunnett's t -test, $p<0.05$, versus control (C)).

shows the mRNA levels, detected by RT-PCR, of β -casein and *PRLr* genes over the period of 21 d after cell seeding. PRL treatment increased the level of β -casein mRNA, and the level was the highest at days 7 and 14. However, at 21 d, the cells showed decreased levels of β -casein. The level of *PRLr* showed a trend of slightly increasing at days 3, 7, and 14 of culture compared to day 1, but it decreased at day 21.

PRL Treatment Increases the Protein Level of β -Casein in MCF-12A Cells To confirm whether activation of the Jak2/STAT5 signaling pathway and production of β -casein in MCF-12A cells was induced by PRL treatment, we determined the protein levels of β -casein, pSTAT5, and STAT5 by Western blot analysis, following PRL treatment of MCF-12A cells. Control cells were cultured in DM for 7 d. For PRL treatment, cells were grown in GM for 1 d and then cultured in DM containing 0.01 μ g/mL or 0.1 μ g/mL of PRL for 6 d. PRL treatment resulted in a concentration-dependent increase in β -casein and pSTAT5. Particularly, the levels of β -casein and pSTAT5 in the cells treated with 0.1 μ g/mL of PRL were increased significantly compared to control cells. However, STAT5 levels increased only slightly with PRL (Fig. 3).

5-HT Treatment Decreases β -Casein Expression in MCF-12A Cells To determine the effect of 5-HT on β -casein expression, we treated cultures with 5-HT for varying periods. The cells were grown for 7 d (in GM for 1 d and in DM containing 0.1 μ g/mL of PRL for 6 d) after seeding and then cultured in the DM containing 0.01 mM or 0.1 mM 5-HT. 5-HT had no influence on cell viability until 96 h after treatment (Fig. 4). RT-PCR was used to quantify β -casein mRNA levels over a time course when the cells were treated with 0.1 mM 5-HT (Fig. 5A). The mRNA level of β -casein was unchanged until 24 h after 5-HT treatment, and then it decreased after a period of exposure to 5-HT, resulting in a significant decrease at 72 h.

Subsequently, we quantified the protein levels of β -casein, pSTAT5, and STAT5 by Western blot analysis of samples from the cells treated for 72 h with 0.1 mM 5-HT. 5-HT treatment decreased the protein levels of β -casein and pSTAT5 in a concentration-dependent manner; in particular, 0.1 mM 5-HT

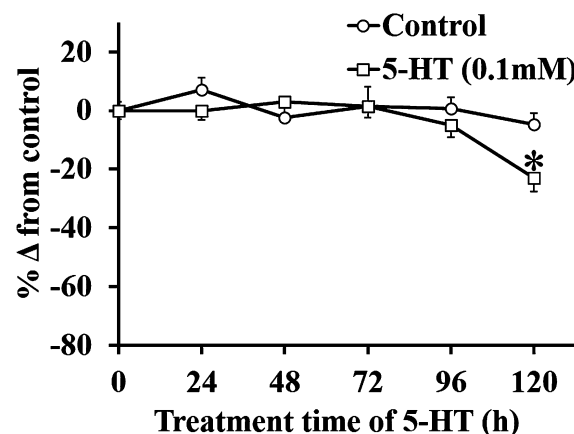


Fig. 4. Viability Curves for MCF-12A Cells Treated with 5-HT

The cells were grown for 7 d (in GM for 1 d and in DM containing 0.1 μ g/mL of PRL for 6 d) after seeding, and then incubated in the DM containing 0.1 mM of 5-HT. Cell viability was measured using Cell Counting Kit-8 at 24, 48, 72, 96, and 120 h of culture. Data were expressed as percent difference of the cells cultured for 7 d (control cells). The values are the means \pm S.E.M. ($n=3-4$). Asterisks (*) indicate significantly different results (Dunnett's test, $p<0.05$, versus value of control cells).

resulted in significant inhibition, compared to control cells. Regarding STAT5, the protein level decreased slightly (Fig. 5B).

DISCUSSION

The objectives of this study were to evaluate whether exogenous 5-HT can suppress β -casein expression produced in human mammary epithelial cells, which are similar to animal cells, and to determine whether 5-HT can attenuate β -casein signaling via the PRLr-Jak2/STAT5 pathway.

First, we conducted some preliminary experiments to functionally characterize MCF-12A cells. We confirmed that 0.1 μ g/mL PRL treatment influences the mRNA levels of β -casein and *PRLr*. Specifically, PRL treatment increased the mRNA level of β -casein, a differentiation marker, and the level was highest at days 7 and 14 of culture. In contrast,

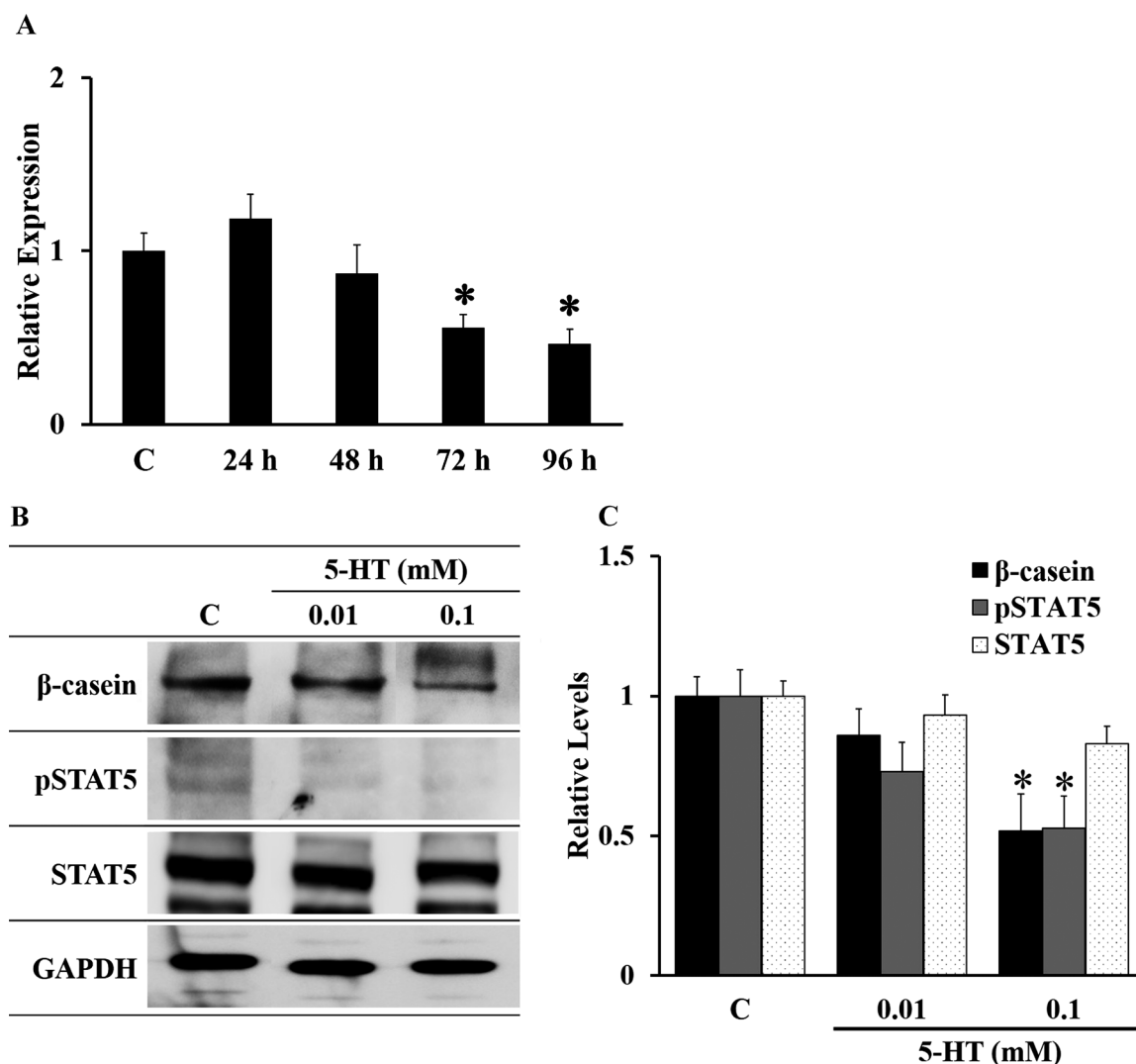


Fig. 5. Effect of 5-HT Treatment on the Expression of β -Casein, pSTAT5, and STAT5 in MCF-12A Cells

A) The relative mRNA levels of β -casein in 5-HT-treated and untreated cells (C) were analyzed by quantitative RT-PCR for the indicated culture time in serum-free DM (containing 0.1 μ g/mL PRL) with 0.1 mM 5-HT, compared to that of the internal standard *GAPDH*. B) The relative protein levels of β -casein, pSTAT5, and STAT5 in 5-HT-treated (5-HT) and untreated cells (C) were analyzed by Western blot. The cells were treated with 0.01 mM or 0.1 mM 5-HT for 72 h. Closed, shaded, and dotted bars indicate the relative levels of β -casein, pSTAT5, and STAT5, respectively. The values are the means \pm S.E.M. ($n=3-4$). Asterisks (*) indicate significantly different results (Dunnett's *t*-test, $p<0.05$ versus control (C)).

PRLr expression was not affected significantly by PRL treatment (Fig. 2). These results demonstrate that MCF-12A cells express *PRLr*, and PRL treatment could induce lactational function of the cells. In addition, we evaluated whether PRL treatment affected the protein level of β -casein and whether activation of the Jak2/STAT5 pathway is responsible for induction of β -casein expression. PRL treatment in MCF-12A cells increased levels of β -casein and pSTAT5 proteins in a concentration-dependent manner, with only a slight increase of STAT5 protein (Fig. 3). Our results suggest that PRL can promote STAT5 phosphorylation, resulting in induction of β -casein expression in MCF-12A.

Second, influences of 5-HT treatment on cell viability and β -casein mRNA expression in MCF-12A cells were evaluated. Although no difference in cell viability was observed until 96 h after 0.1 mM 5-HT treatment (Fig. 4), inhibition of β -casein expression was observed to be time-dependent (Fig. 5A). Additionally, treatment with 0.1 mM 5-HT for 72 h decreased protein levels of β -casein and pSTAT5, with a very slight decrease in STAT5 (Fig. 5B). These results suggest that

exogenous 5-HT can inhibit STAT5 phosphorylation, resulting in inhibition of β -casein expression. A previous paper reported that treatment with 0.2 mM 5-HT for 48 h decreases mRNA expression of genes encoding milk proteins, including β -casein and α -lactalbumin, in primary bovine mammary epithelial cells.¹⁴⁾ Since our findings are similar to these results, we conclude that a 5-HT-regulated mechanism is likely present in MCF-12A cells.

Mammary gland involution is induced by the absence of a suckling stimulus, resulting in decline of lactation function.¹⁷⁾ During involution, mammary epithelial cells cause disruption of tight junctions, cell shedding, and cell apoptosis. Treatment with 2.5 mM 5-HT for 48 h decreases zonula occluden 1 and 2 proteins, which are tight junction scaffolding proteins, via the 5-HT7 receptor in MCF-10A human mammary epithelial cells.^{17,18)} In addition, treatment with 0.1 mM 5-HT for 72 h causes cell shedding and an increased level of caspase-3 in MCF-10A cells, suggesting that 5-HT induces apoptosis in mammary epithelial cells.¹⁹⁾ Although we did not determine levels of the 5-HT7 receptor or occurrence of apoptosis in this

study, the decrease in β -casein protein upon 5-HT treatment is likely induced *via* signaling through the 5-HT₇ receptor, accompanied by apoptosis. Furthermore, a 5-HT transporter has been detected in human mammary epithelial cells,²⁰⁾ and levels of tryptophan hydroxylase 1 (TPH1), a rate-limiting enzyme in 5-HT biosynthesis, are elevated during pregnancy and lactation in mouse and bat mammary epithelial cells.^{14,21)} Fluoxetine, an SSRI that perturbs serotonin balance by binding to the serotonin reuptake transporter within mammary glands, inhibits β -casein expression in primary bovine mammary epithelial cells.¹⁶⁾ To elucidate 5-HT-mediated mechanisms in MCF-12A cells, it will be important to evaluate not only these genes but also SSRI action on the cells.

The regulation of 5-HT activity within mammary glands is multifaceted, and 5-HT action is modulated by a balance between synthesis and degradation. During lactation, mammary epithelial cells activate 5-HT synthesis by TPH1.¹⁴⁾ TPH1 catalyzes the rate-limiting step in the biosynthesis of 5-HT within mammary glands. 5-HT synthesized in mammary glands is released into both apical (milk) and basolateral space by a vesicular monoamine transporter.²²⁾ 5-HT released into milk is incorporated by the serotonin reuptake transporter (SERT), which is expressed at apical membranes,¹⁷⁾ and then it is degraded by the monoamine oxidase A enzyme.²²⁾ Suckling maintains 5-HT at low levels in milk. If milk letdown does not occur, thus causing milk stasis, sustained elevation of 5-HT initiates involution.²²⁾ Since SERT transports 5-HT, SERT is likely associated with these lactation events; however, its exact mechanism of action is unknown.

In conclusion, we showed that exogenous 5-HT decreased β -casein expression in MCF-12A human mammary epithelial cells and that 5-HT was responsible for inhibition of STAT5 phosphorylation, resulting in a decline in lactational function.

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