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Smoking-induced suppression of β-casein in milk is associated with an increase in miR-210-5p expression in mammary epithelia

Takeshi Chiba [a,b,*], Akira Takaguri [c,d], Toshiyasu Mikuma [e], Toshimi Kimura [a,b], Tomoji Maeda [f]

[a] Department of Pharmacy, Juntendo University Hospital, 3-1-3 Hongo, Bunkyo-ku, Tokyo, 113-8431, Japan
[b] Laboratory of Clinical Pharmacology, Faculty of Pharmacy, Juntendo University, 6-8-1 Hinode, Urayasu-shi, Chiba, 279-0013, Japan
[c] Department of Pharmacology, Faculty of Pharmaceutical Sciences, Hokkaido University of Science, 15-4-1, Maeda 7-jo, Teine-ku, Sapporo-shi, Hokkaido, 006-8585, Japan
[d] Creation Research Institute of Life Science in KITA-no-DAICHI, Hokkaido University of Science, 15-4-1, Maeda 7-jo, Teine-ku, Sapporo-shi, Hokkaido, 006-8585, Japan
[e] Department of Physical and Analytical Sciences, Nihon Pharmaceutical University, 10281 Komuro, Ina-machi, Kitaadachi-gun, Saitama, 362-0806, Japan
[f] Department of Clinical Pharmacology and Pharmaceutics, Nihon Pharmaceutical University, 10281 Komuro, Ina-machi, Kitaadachi-gun, Saitama-shi, 362-0806, Japan

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ABSTRACT

Smoking during lactation harmfully affects the amount and constituents of breast milk. Infants who consume breast milk containing miR-210-5p may have a higher risk of brain-related diseases. We investigated whether smoking during lactation decreases β-casein concentrations in milk and whether miR-210-5p expression is involved in smoking-induced β-casein suppression. During lactation, maternal CD1 mice were exposed to cigarette smoke (1.7 mg of tar and 14 mg of nicotine) in a smoke chamber for 1 h twice/day for five consecutive days. Control mice were placed in an air-filled chamber equivalent in size to the smoke chamber, with maternal separation times identical to those of the smoked mice. Maternal exposure to smoke during lactation significantly decreased β-casein expression in the mammary epithelia of smoked mice compared to that of the control mice. Signal transducer and activator transcription 5 (STAT5) and phosphorylated STAT5 (pSTAT5) are transcription factors involved in β-casein expression. In the mammary epithelia of smoked mice, the pSTAT5 and STAT5 levels were significantly lower, and miR-210-5p expression was significantly higher than that of the control mice. The β-casein, pSTAT5, and STAT5 protein levels of miR-210-5p mimic-transfected human mammary epithelial MCF-12A cells were significantly lower than those of control siRNA-transfected cells. These results indicate that smoke exposure led to an increase in miR-210-5p expression in mammary epithelium and a decrease in pSTAT5 and β-casein protein levels through the inhibition of STAT5 expression. Moreover, nicotine treatment decreased β-casein protein levels and increased miR-210-5p expression in non-malignant human mammary epithelial MCF-12A cells in a concentration-dependent manner, demonstrating that nicotine significantly affects the β-casein and miR-210-5p levels of breast milk. These results highlight the adverse effects of smoking on breast milk, providing essential information for healthcare professionals and general citizens.

1. Introduction

Breastfeeding provides many benefits to both mother and infant. Breastfed infants have health advantages, including a reduced risk of sudden infant death syndrome (SIDS) and reduced morbidity caused by lower respiratory infection and asthma [1–3]. Breastfeeding prevents depression in mothers and decreases the incidence of breast and ovarian cancers [4–7].

Adult mammary gland characteristics are altered by numerous hormones and local factors during pregnancy, resulting in milk production during lactation. Prolactin (PRL) is an anterior pituitary hormone essential for mammary epithelial differentiation and milk production

Abbreviations: ANOVA, analysis of variance; CS, cigarette smoke; cel-miR-39, *Caenorhabditis elegans* miR-39-3p; DM, differentiation medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM, growth medium; HRP, horseradish peroxidase; hEGF, human recombinant epithelial growth factor; Jak2/STAT5, Janus kinase 2/signal transducer and activator transcription 5; miRNAs, microRNAs; nAChRs, nicotine acetylcholine receptors; pSTAT5, phosphorylated STAT5; PRL, prolactin; STAT5, signal transducer and activator transcription 5; SIDS, sudden infant death disease syndrome; Snap25, synaptosomal-associated protein of 25 kDa.

* Corresponding author. Department of Pharmacy, Juntendo University Hospital, 3-1-3, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.
E-mail address: t.chiba.da@juntendo.ac.jp (T. Chiba).

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[8]. Signaling via the Janus kinase 2/signal transducer and activator transcription 5 (Jak2/STAT5) pathway is involved in β-casein expression [9]. β-casein is a milk protein and an important marker of mammary epithelial cell differentiation [10]. PRL activates the Jak2/STAT5 pathway via the PRL receptor and includes Jak2 activation followed by STAT5 phosphorylation [11–14].

Nicotine is the principal tobacco alkaloid and a major psychoactive component of tobacco smoke [15]. Nicotine from tobacco smoke is rapidly absorbed, and maternal nicotine is transferred from plasma to breast milk [16]. Nicotine is rapidly metabolized in the liver, most of which is converted into the major metabolite cotinine [17]. The half-life of cotinine is markedly longer than that of nicotine (20 h vs. 81 min); therefore, the cotinine concentration is more frequently used as a biomarker for tobacco smoke exposure [15].

Smoking during lactation harmfully affects the amount and constituents of the milk produced. A previous study reported that the daily amount of milk (≤2 weeks after birth) was 406 ± 262 mL/day in smoking mothers compared to 514 ± 338 mL/day in the control group (non-smoking mothers [18]. A clinical study showed that the amount of total protein in the breast milk of smoking mothers was significantly lower than that of non-smoking mothers [19]. Furthermore, nicotine treatment significantly decreased β-casein expression in primary mammary epithelial cells obtained from lactating mice [16]. These results suggest that smoking during lactation may negatively affect the growth and development of infants by altering the milk yield and constituents.

MicroRNAs (miRNAs) are short noncoding RNAs found in body fluids, including blood, saliva, urine, and breast milk. In addition, miRNAs bind to target mRNAs and regulate their expression by inhibiting their translation [20,21]. Fujita et al. showed that the miR-210 expression levels are significantly higher in the lung tissues of smokers than in those of non-smokers [22]. Furthermore, they reported that smoking-induced increases in miR-210 expression levels promote pulmonary fibroblast differentiation and may contribute to airway remodeling in chronic obstructive pulmonary disease [22]. These results suggest a close relationship between smoking and miR-210 expression. The miRNAs found in breast milk are synthesized in the mammary epithelial cells [23]. A previous study showed that miR-210 (both miR-210-3p and miR-210-5p) is present in breast milk [24]. miR-210-5p targets STAT5A mRNA and regulates Jak2 signaling [25,26], suggesting that mammary-derived miR-210-5p may be associated with the regulation of β-casein expression via Jak2/STAT5 pathway inhibition in mammary epithelial cells. However, to the best of our knowledge, no studies have investigated whether smoking during lactation affects mammary-derived miR-210-5p expression. Additionally, the relationship between the smoking-induced changes in mammary-derived miR-210-5p expression and β-casein concentration in breast milk has not been explored. This study investigated the influence of smoking on the β-casein concentration in milk and whether this change in β-casein concentration is involved in the expression of mammary-derived miR-210-5p.

2. Materials and methods

2.1. Animal study

Pregnant female CD1 mice (14 days of gestation) were purchased from Charles River Laboratories (Wilmington, MA, USA). All mice were maintained in an air-conditioned animal room (temperature: 23 ± 2 °C; relative humidity: 55 ± 15 %; 12-h light/dark cycle). Commercially available filter-tipped cigarettes, including 1.7 mg of tar and 14 mg of nicotine per cigarette (Hi-lite, Japan Tobacco, Inc., Tokyo, Japan), were used in this study. During lactation, maternal mice were either exposed to cigarette smoke (CS) produced from one cigarette in a smoke chamber (500 mm [W] × 350 mm [D] × 300 mm [H]; three animals/chamber; Fig. 1) or exposed to room air. Smoked mice were subjected to CS for 1 h twice/day (9 a.m. and 3 p.m.) for five consecutive days (7–11 days postpartum). Control mice were placed in an air-filled chamber of the same size as the smoke chamber, with maternal separation times identical to those of the smoked mice. Control and smoked mice did not receive simple general smoke. The inflow rate from the syringe to the chamber was approximately 200 mL/min, and it took approximately 5 min to complete cigarette combustion. Total particle concentration was maintained at 14 mg/m^3 from the time at which cigarette burning was terminated until smoke exposure was concluded. Milk, mammary epithelium, lung tissue, and whole blood samples were collected from the mice after exposure to CS on day 11 postpartum. Milk was collected as described previously [27,28]. After milk collection, mammary epithelial and lung tissues were collected from the mice and frozen in liquid nitrogen. Whole blood samples were collected via cardiocentesis, and plasma samples were obtained by centrifugation immediately after whole blood collection. The collected milk and plasma samples were preserved at −80 °C until analysis.

All procedures pertaining to the animals used in this study were performed in compliance with the “Guidelines for Proper Conduct of

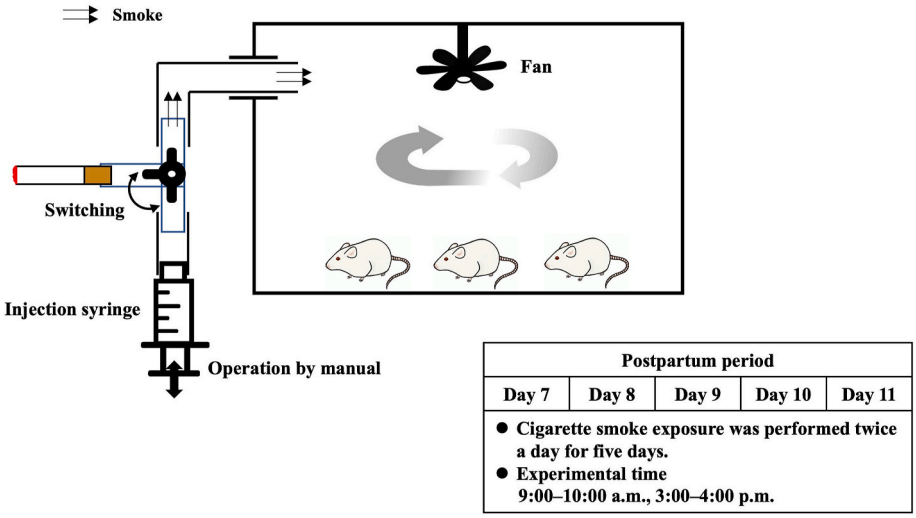

Fig. 1. The smoke chamber model. Maternal mice were either exposed to cigarette smoke (CS) produced from one cigarette in a smoke chamber (500 mm [W] × 350 mm [D] × 300 mm [H]; three animals/chamber. Smoked mice were subjected to CS for 1 h twice/day (9 a.m. and 3 p.m.) for five consecutive days (7–11 days postpartum).

Animal Experiments" (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care Committee of Hokkaido University of Science (Approval No. 2022-012). All efforts were made to minimize animal suffering.

2.2. Quantitative polymerase chain reaction analysis

Quantitative polymerase chain reaction (PCR) analysis of miRNA expression in milk, mammary epithelium, lung tissue, and cultured cells was performed as previously described [27,28]. Briefly, total RNA was extracted to evaluate miRNA expression using the miRNeasy Serum/Mini Kit (Qiagen, Hilden, Germany) or the miRNeasy Mini Kit (Qiagen). *Caenorhabditis elegans* miR-39-3p (cel-miR-39; Qiagen) was added to milk as a spike-in control. cDNA was synthesized using the TaqMan miRNA Reverse Transcription Kit (Thermo Fisher Scientific, MA, USA). Cycle numbers of miR-210-5p, cel-miR-39, and U6 short hairpin RNA (shRNA; the internal control) were obtained using the 7500 Fast Real-time PCR system, TaqMan Universal Master Mix II, and commercially available predesigned primer and probe sets [mmu-miR-210-5p:462444_mat (for mouse), hsa-miR-210-5p: 467276_mat (for human), cel-miR-39:000200, and U6 shRNA:001973; Thermo Fisher Scientific]. The expression of miR-210-5p in milk was normalized to that of cel-miR-39. The miR-210-5p expression levels in the mammary epithelium, lung tissues, and cultured cells were normalized to those of U6 shRNA.

Total RNA was extracted to evaluate mRNA expression using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the HiCapacity RNA-to-cDNA Kit (Thermo Fisher Scientific). The cycle numbers of β-casein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from the 7500 Fast Real-time PCR system, TaqMan Universal Master Mix II, and commercially available predesigned primer and probe sets (*β-casein*: Mm04207885_m1, *GAPDH*: Mm99999915_g1; Thermo Fisher Scientific). The β-casein expression level was normalized to that of GAPDH. All samples were analyzed in triplicate, and the data are presented as mean relative levels.

2.3. Western blot analysis

Protein extraction from tissues or cultured cells was performed using the T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific) or M-PER™ Mammalian Protein Extraction Kit (Thermo Fisher Scientific), respectively. Lysates were exposed to electrophoresis on a gradient polyacrylamide gel (e-PAGEL 5–20 %; ATTO, Tokyo, Japan) and then transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with Block Ace (KAC, Kyoto, Japan). The primary antibodies used in this study were mouse anti-β-casein (1:250; NB100-2720; Novus Biologicals, Littleton, CO, USA), rabbit anti-STAT5 (1:1000; ab32043; Abcam, Cambridge, UK), rabbit anti-phosphorylated STAT5 (pSTAT5; 1:1000; ab98338; Abcam), and mouse anti-GAPDH (1:3000; Merck KGaA, Darmstadt, Germany). The secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse antibodies (1:4000; Cell Signaling Technology, Danvers, MA, USA) were incubated with the Amersham ECL Prime Western Blotting Detection Reagents (Cytiva, Marlborough, MA, USA), and immunoreactive bands were visualized using the ChemiDoc Imaging system (Bio-Rad Laboratories, Hercules CA, USA). Band intensities were analyzed using the ImageJ software (National Institute of Health, Bethesda, MD, USA). β-casein, pSTAT5, and STAT5 were detected in the same blot as GAPDH, and each signal was normalized to that of GAPDH.

2.4. Cell culture

Non-malignant human mammary epithelial MCF-12A cells (American Type Culture Collection, Manassa, VA, USA) were seeded on Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA)-coated plates at a density of 0.6×10^5 cells/cm^2 and cultured in growth medium (GM; Dulbecco's modified Eagle medium/Ham's F12 Nutrient Mixture; 1:1; Sigma-Aldrich, St. Louis, MO, USA). The GM was supplemented with 10 μg/mL human insulin (Sigma-Aldrich), 0.5 μg/mL hydrocortisone (Sigma-Aldrich), 20 ng/mL human recombinant epithelial growth factor (hEGF; BD Biosciences), 5 % horse serum (Sigma-Aldrich), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich). The GM was changed to a differentiation medium (DM) modified by the addition of human PRL (0.1 μg/mL, FUJIFILM Wako Pure Chemical Co., Tokyo, Japan) and removal of hEGF at 24 h after cell seeding. Nicotine (Sigma-Aldrich) was added to the DM at a final concentration of 1, 30, and 100 μM, and cells were subjected to these treatments for 72 h.

2.5. Transient transfection of the miR-210 mimic into MCF-12A cells

MCF-12A cells were transfected with the control or miR-210-5p mimic (Syn-hsa-miR-210-5p; MSY0026475; Qiagen) at a final concentration of 40 nmol/L in DM using the HiPerfect Transfection Reagent (Qiagen). The miR-210-5p mimic sequence used was 5′-AGCCCCUGCCCACCGCACACUG-3′. AllStars negative control siRNA labeled with Alexa Fluor 488 (Qiagen) was used as the control miRNA mimic, as recommended by the manufacturer.

2.6. Enzyme-linked immunoassay (ELISA)

Milk obtained from mice was centrifuged at 4 °C and 10000×*g* for 10 min. The intermediate layer between the lipid layer and precipitate was used to determine β-casein concentration using the Mouse CSN2/β-casein ELISA Kit (LifeSpan Bioscience, Inc., Seattle, WA, USA). The assay was performed according to the manufacturer's instructions.

2.7. Determination of plasma nicotine and cotinine in mice

Plasma (100 μL) obtained from each mouse was diluted by water (300 μL) containing the internal standards of nicotine and cotinine (nicotine-d3, Hayashi Pure Chemical Ind., Ltd., Osaka, Japan; cotinine-d_3, Merck KGaA). The samples were deproteinized by mixing with 25 % trichloroacetic acid (100 μL), vortexed, and centrifuged at 8000×*g* for 10 min. The supernatants were mixed with 1 M sodium hydroxide (200 μL). The mixture was loaded onto an Oasis HLB solid extraction cartridge (Waters, Milford, MA, USA) preconditioned with methanol and water. The cartridge was washed with a mixture (9:1) of 10 mM phosphate buffer (pH 9) and methanol, and the desired fraction was extracted with methanol containing 1 % formic acid. The eluted fraction was dried using nitrogen gas and the residue was reconstituted in acetonitrile (200 μL). A sample (5 μL) of this solution was removed and analyzed.

Plasma nicotine and cotinine concentrations were determined via liquid chromatography-tandem mass spectrometry (LC/MS/MS; AB SCIEX QTRAP® 6500 system; AB Sciex, Framingham, MA, USA). An Atlantis HILIC Silica column (length: 100 mm, internal diameter: 2.1 mm, particle size: 3 μm; Waters) was used for separation at a temperature of 40 °C. The mobile phase consisted of an aqueous 10 mM ammonium acetate solution (A) and acetonitrile containing 0.1 % formic acid (B). The gradient program of the mobile phase was as follows: 90 % B for 0–3 min; linear gradient: 90–50 % B for 3–10 min; isocratic elution: 50 % B for 10–12 min; re-equilibration: 90 % B for 8 min. The total run time was 20 min.

The turbo ion spray interface was operated in the positive ion mode (5500 V) under the following operating conditions: ion source temperature, 600 °C; ion source gas 1, 60 psi; ion source gas 2, 60 psi; curtain gas, 30 psi; and collision gas, 10 psi. The mass spectrometer was operated in multiple reaction monitoring mode. The signal output was simultaneously monitored for two separate ion pairs for nicotine (*m/z* 163.1/117.1, and *m/z* 163.1/130.1) and cotinine (*m/z* 177.1/80.1, and *m/z* 177.1/98.1), and single ion pairs for nicotine-d_3 (*m/z* 166.1/130.1) and cotinine-d_3 (*m/z* 180.1/80.1).

2.8. Statistical analysis

The Student's *t*-test was used to compare the data between the two groups. For comparisons between multiple groups, data were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test to compare each group with the control. All statistical analyses were conducted using JMP software (SAS Institute, Cary, NC, USA). Data are presented as means ± standard error of the mean. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Plasma nicotine and cotinine concentrations in mice

To confirm the extent of exposure to CS in the smoked mice, plasma nicotine and cotinine concentrations in the smoked and control mice were determined using LC/MS/MS. Plasma nicotine concentrations in the smoked mice were significantly higher than those of the control mice (Fig. 2a; smoked mice: 11.6 ± 1.31 ng/mL vs. control mice: 1.06 ± 0.08 ng/mL, $P < 0.05$). Plasma cotinine was detected in the smoked mice, but it was not detected in the control mice (Fig. 2b; smoked mice: 7.64 ± 0.94 ng/mL, $P < 0.05$).

3.2. Influence of smoking on β-casein levels in the milk and mammary epithelium of mice

ELISA was used to investigate the influence of smoking on β-casein concentrations in milk. In the smoked mice, the β-casein concentration in milk was significantly lower than that of the control mice ($P < 0.05$; Fig. 3a). Additionally, β-casein mRNA expression in the mammary epithelium of smoked mice was significantly lower than that of the control mice ($P < 0.05$; Fig. 3b). The protein levels of pSTAT5 and STAT5 in the mammary epithelia of smoked mice were significantly lower than those in the mammary epithelia of control mice ($P < 0.05$; Fig. 3c and d). However, there was no significant difference in the pSTAT5/STAT5 ratio between the two groups (Fig. 3e, $P = 0.085$).

3.3. Influence of smoking on miR-210-5p expression in the lung tissue, milk, mammary epithelium, and plasma of mice

To investigate the relationship between smoking and miR-210-5p expression, miR-210-5p expression in the lung tissue, milk, mammary epithelium, and plasma of smoked and control mice was evaluated using quantitative PCR. Expression of miR-210-5p in the lung tissues of smoked mice was significantly higher than that in the lung tissues of control mice ($P < 0.05$; Fig. 4a). Additionally, miR-210-5p expression in milk and mammary epithelium was significantly higher than that in the control mice ($P < 0.05$; Fig. 4b and c). Plasma miR-210-5p expression did not differ significantly between the smoked and control mice ($P < 0.05$; Fig. 4d).

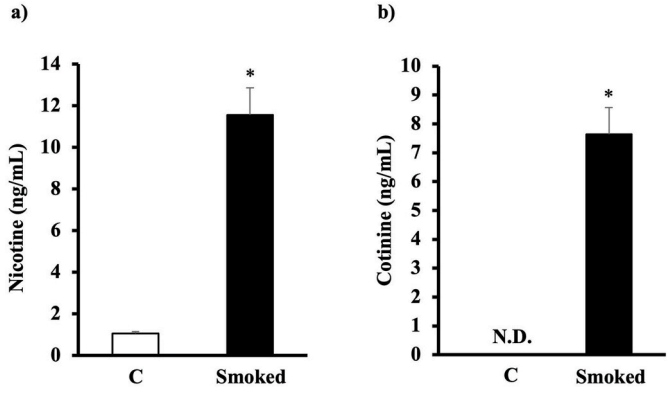

Fig. 2. Plasma nicotine and cotinine concentrations in the smoked mice. Plasma nicotine a) and cotinine b) concentrations in the smoked and control mice determined via LC/MS/MS. Data are expressed as the mean ± SE (n = 3). N.D.: not detected. *$P < 0.05$, Student's *t*-test, to control (c) mice.

3.4. Expression of β-casein in miR-210-5p mimic-transfected MCF-12A cells

To investigate the association between miR-210-5p expression and β-casein expression, the protein levels of β-casein, pSTAT5, and STAT5 in MCF-12A cells transfected with the miR-210-5p mimic were evaluated via western blotting. The relative miR-210-5p expression levels in cells transfected with the miR-210-5p mimic were significantly higher than those in cells transfected with the control siRNA ($P < 0.05$; Fig. 5a). Additionally, the protein levels of β-casein, pSTAT5, and STAT5 in cells transfected with the miR-210-5p mimic were significantly lower than those in cells transfected with the control siRNA ($P < 0.05$; Fig. 5b, c, and 5d). However, there was no significant difference in the pSTAT5/STAT5 ratio between the two groups (Fig. 5e, $P = 0.132$).

3.5. Influence of nicotine treatment on β-casein and miR-210-5p expression in MCF-12A cells

To evaluate whether smoking-derived nicotine affects β-casein and miR-210-5p expression in the mammary epithelia of mice, β-casein or miR-210-5p expression in MCF-12A cells treated with nicotine was assessed using Western blot or quantitative PCR analysis, respectively. Nicotine treatment decreased the β-casein protein levels in a concentration-dependent manner. In cells treated with 30 and 100 μM of nicotine, the β-casein protein levels decreased significantly when compared with those in the control cells ($P < 0.05$; Fig. 6a). In contrast, nicotine treatment increased the miR-210-5p expression levels in a concentration-dependent manner. In cells treated with 30 and 100 μM of nicotine, miR-210-5p expression increased significantly compared to that in the control cells ($P < 0.05$; Fig. 6b).

4. Discussion

This study investigated whether smoking during lactation decreases β-casein concentrations in milk and whether miR-210-5p expression is involved in the smoking-induced suppression of β-casein.

First, to confirm CS exposure in smoked mice, the plasma nicotine and cotinine concentrations in smoked and control mice were evaluated via LC/MS/MS. Smoking-derived nicotine is metabolized by CYP2A6 in the liver and converted to a major metabolite, cotinine, which is less toxic than nicotine [29]. The plasma concentration of cotinine, which has a long half-life (approximately 20 h), is used as a biomarker of CS exposure [15]. Both nicotine and cotinine were detected in the plasma of mice that were exposed to tobacco smoke (Fig. 2a and b). Additionally, cotinine was detected only in the plasma of the smoked mice (Fig. 2b). These results imply that the CS exposure experiment using our smoke chamber was successfully implemented in this study. However, low levels of nicotine were detected in the plasma of the control mice in this study (Fig. 2a). When smoked mice were returned from the smoke chamber to the breeding cages after CS exposure, CS in the smoke chamber may have leaked into the room air; therefore, detection of nicotine in the plasma of control mice may have been due to exposure to nicotine that had diffused into the room air.

Second, the β-casein levels in milk and mammary epithelia obtained from the smoked mice were evaluated. Smoking significantly decreased β-casein concentrations in milk and significantly reduced β-casein mRNA levels in the mammary epithelium (Fig. 3a and b). Additionally, smoke exposure significantly decreased the protein levels of pSTAT5 and STAT5, which are transcription factors of the Jak2/STAT5 pathway involved in β-casein expression in the mammary epithelium [9] (Fig. 3c and d). Kobayashi et al. reported that treating primary mammary

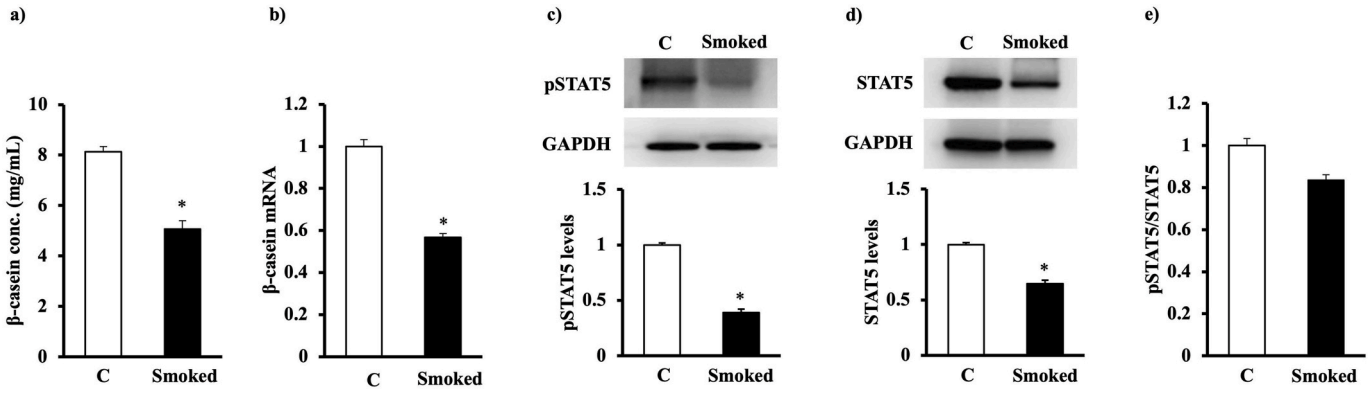

Fig. 3. Influence of smoking on β-casein levels in milk and the mammary epithelia of mice. **a**) β-casein concentrations in milk determined by ELISA. **b**) Relative levels of *β-casein* mRNA expression in mammary epithelium analyzed by quantitative PCR. The level of *β-casein* mRNA is normalized to that of *GAPDH*. The relative protein levels of pSTAT5 **c**) and STAT5 **d**) and the pSTAT5/STAT5 ratio **e**) in the mammary epithelium analyzed by Western blot. The levels of pSTAT5 and STAT5 are normalized to those of GAPDH. Data are expressed as the mean ± SE (n = 3). *$P < 0.05$, Student's *t*-test, compared to control (C) mice.

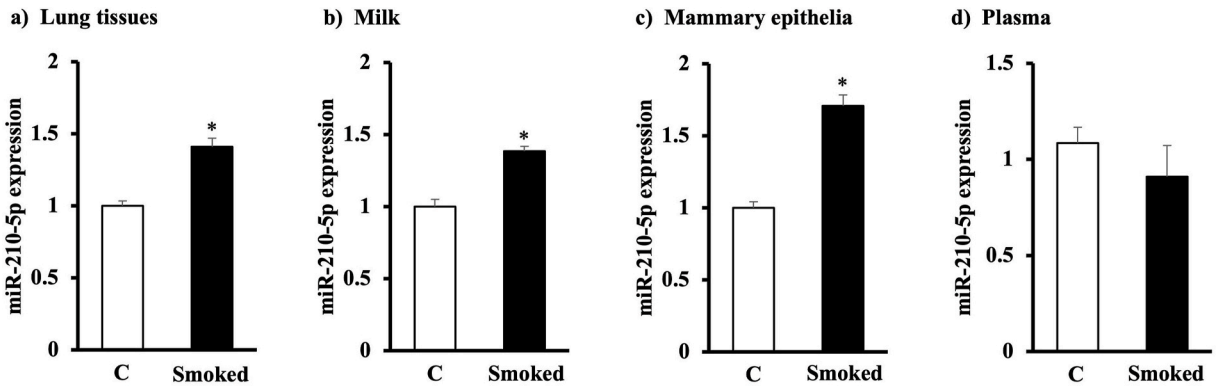

Fig. 4. Influence of smoking on miR-210-5p expression in lung tissues, milk, mammary epithelia, and plasma in mice. Relative levels of miR-210-5p expression in lung tissue (**a**), milk (**b**), mammary epithelium (**c**), and plasma (**d**) are analyzed via quantitative PCR. The level of miR-210-5p expression in the lung tissue and mammary epithelium is normalized to that of the endogenous control U6 shRNA. The level of miR-210-5p expression in milk and plasma is normalized to that of spike-in-control cel-miR-39. Data are expressed as the mean ± SE (n = 3). *$P < 0.05$, Student's *t*-test, compared to control (C) mice.

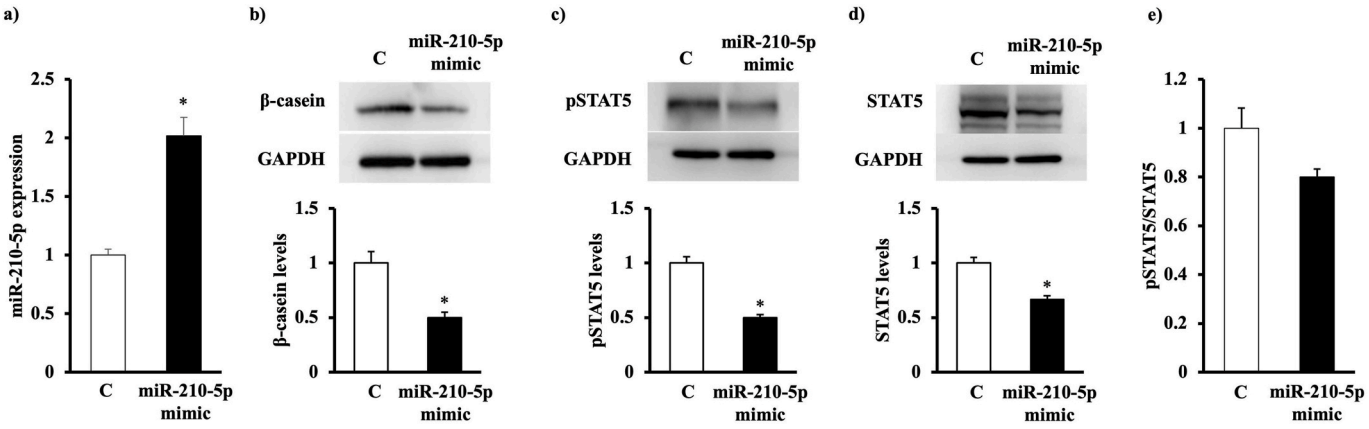

Fig. 5. Expression of β-casein in miR-210-5p mimic-transfected MCF-12A cells, The relative level of miR-210-5p expression (**a**) in MCF-12A cells transfected with the miR-210-5p mimic is analyzed using quantitative PCR. The level of miR-210-5p expression in cells is normalized to that of the endogenous control U6 shRNA. The relative protein levels of β-casein **b**), pSTAT5 **c**), and STAT5 **d**), and the pSTAT5/STAT5 ratio **e**) in MCF-12A cells transfected with the miR-210-5p mimic and analyzed by Western blot. Protein levels are normalized to those of the endogenous control GAPDH. Data are expressed as the mean ± SE (n = 3). *$P < 0.05$, Student's *t*-test, compared to control (C) cells.

epithelial cells from lactating mice with 33 μM of nicotine for 3 days reduced intracellular β-casein expression. Additionally, they demonstrated that treating these cells with 10–100 μM nicotine for 3 days decreased the concentration of secreted β-casein in the culture fluid [16]. Bachour et al. showed that the amount of total protein in breast milk obtained from smokers was significantly lower than that of non-smokers [19]. These findings support our observations. Furthermore, our results show that smoking inhibited STAT5 phosphorylation

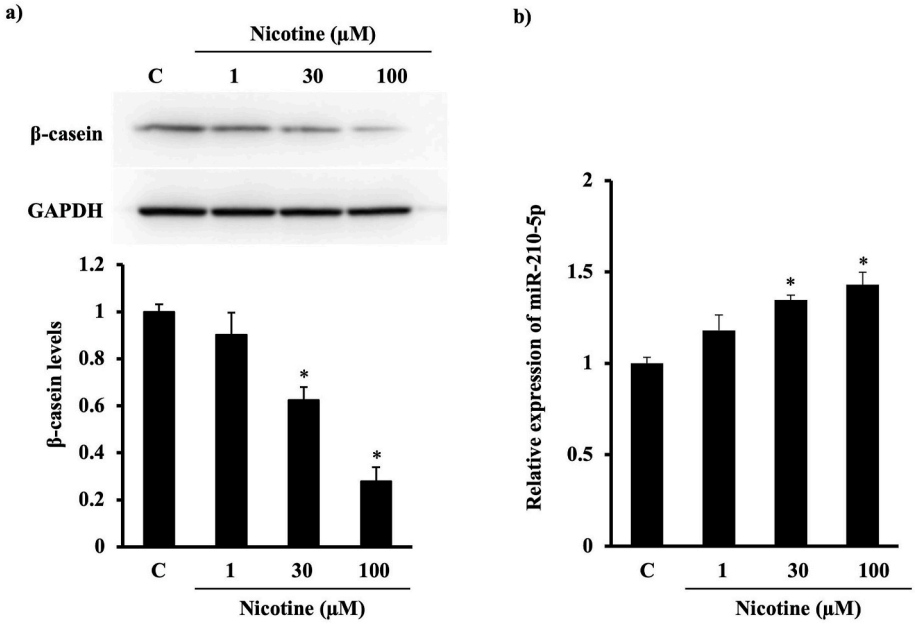

Fig. 6. Influence of nicotine treatment on the β-casein and miR-210-5p expression levels in MCF-12A cells. The relative level of β-casein protein **a**) and the relative miR-210-5p expression level **b**) in MCF-12A cells treated with nicotine (1, 30, and 100 μM) for 72 h analyzed via Western blot or quantitative PCR, respectively. The level of β-casein is normalized to that of GAPDH. The level of miR-210-5p is normalized to that of the endogenous control U6 shRNA. Data are expressed as the mean ± SE (n = 3). *$P < 0.05$, one-way ANOVA followed by the Dunnett's test, compared to control (C) cells.

and β-casein expression in the mammary epithelium and that these changes were involved in the β-casein concentration reduction observed in milk.

Third, to investigate whether miR-210-5p expression in the milk and mammary epithelium was related to smoking during lactation, miR-210-5p expression levels in the lung tissues, mammary epithelia, and plasma of smoked mice were evaluated. Fujita et al. reported that smoking induces lung-derived miR-210 expression in smokers [22]. In this study, we confirmed a significant increase in miR-210-5p expression in the lung tissues of smoked mice compared to that of the control mice (Fig. 4a). Our experimental animal model, therefore, exhibited a phenomenon similar to that observed in humans. Additionally, our results showed that smoke exposure significantly increased miR-210-5p expression in milk and mammary epithelia, although it did not significantly change miR-210-5p expression in the plasma. These results indicate that the smoking-induced increase in miR-210-5p expression in the mammary epithelium is unlikely to be associated with an increase in lung-derived miR-210-5p. As milk-derived miRNAs are synthesized by mammary epithelial cells, our observations suggest that the smoking-induced increase in miR-210-5p expression in milk was due to an increase in mammary-derived miR-210-5p expression.

Subsequently, we investigated the association between the smoking-induced decrease in β-casein concentration in milk and the increase in mammary-derived miR-210-5p expression. The protein levels of β-casein, pSTAT5, and STAT5 in miR-210-5p-transfected MCF-12A cells were significantly lower than those in the control cells (Fig. 5a, b, and 5c). These results are similar to those observed in our animal model of smoking. Our results showed that the smoking-induced decrease in β-casein concentrations in milk was associated with an increase in mammary-derived miR-210-5p expression. STAT5 comprises of STAT5A and STAT5B. STAT5A is involved in β-casein expression via the Jak2/STAT5 signaling pathway [9]. A dual-luciferase reporter assay using human dermal fibroblasts showed that miR-210-5p directly binds to the 3′-UTR of STAT5A, indicating that miR-210-5p targets STAT5A [26]. This suggests that the reduced STAT5 levels in cells transfected with the miR-210-5p mimic may be due to a decrease in the STAT5A levels. Additionally, although pSTAT5/STAT5 levels in the mammary epithelium of smoking mice and MCF-12A cells transfected with miR-210-5p mimics were not significantly different from those of control mice and control cells, they tended to be lower (Figs. 3e and 5e). These observations suggest that miR-210-5p may suppress not only STAT5 inhibition but also STAT5 phosphorylation in mammary epithelia. Further studies should be performed to investigate whether miR-210-5p inhibits STAT5 phosphorylation. Taken together, these observations indicate that miR-210-5p expression induced by smoking inhibits the levels of β-casein and pSTAT5 via a decline in STAT5, resulting in the decreased β-casein concentrations observed in maternal milk.

We also evaluated whether the smoking-induced increase in miR-210-5p expression in the mammary epithelium was associated with the major component of smoking (nicotine). Nicotine treatment increased miR-210-5p expression and decreased β-casein expression in a concentration-dependent manner in MCF-12A cells (Fig. 6a and b). These results demonstrate that the smoking-induced increases in miR-210-5p expression and decreases in β-casein expression in mammary epithelia were caused by the presence of nicotine in mice. Taken together, smoking-derived nicotine induced miR-210-5p expression in mammary epithelia, and the increased levels of miR-210-5p decreased the protein levels of pSTAT5 and β-casein via STAT5 inhibition, thereby reducing the β-casein concentrations in milk.

Nicotine acetylcholine receptors (nAChRs) are composed of nine α subunits (α2–α10) and two β subunits (β2 and β4) [30]. In the present study, we did not investigate the expression profiles of nAChRs in MCF-12A cells. Non-malignant human mammary epithelial HBL-100 cells express four nAChR subunits: α4, α5, α9, and β2 [31]. Additionally, the lactating mouse mammary epithelium expresses seven nAChR subunits: α2, α3, α4, α6, α7, α2, and β4 [16]. These reports show that the α4 subunits expressed in mammary epithelial cells are common between humans and mice. This receptor may be associated with the nicotine-induced increase in miR-210 expression. Further studies are required to identify the nAChRs associated with nicotine-induced increases in miR-210 expression in mammary epithelial cells.

Nakajima et al. reported a maximum plasma concentration of nicotine of approximately 10 ng/mL after smoking one cigarette [32]. In this

study, the plasma nicotine concentration in smoked mice was 11.5 ng/mL, which was similar to physiological nicotine concentrations in human smokers. However, directly comparing plasma nicotine concentrations between humans and mice poses challenges owing to differences in nicotine sensitivity across species. Subsequent investigations will aim to ascertain the validity of our *in vivo* model in replicating human smoking conditions. Moreover, in this study, a concentration of 30 μM nicotine caused a decrease in β-casein expression and an increase in miR-210-5p expression in MCF-12A cells (Fig. 2a and b). Notably, this treatment concentration greatly exceeded the physiological plasma concentrations of nicotine in human smokers. Tobacco smoke contains more than 1000 chemical substances including nicotine [33]. These substances include hazardous compounds such as benzo[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-diozin (TCDD) [33]. Collin et al. demonstrated that TCDD reduces pSTAT5 and β-casein protein levels by activating the aryl hydrocarbon receptors in mouse mammary epithelia [34]. This suggests that the observed decrease in β-casein expression in the mammary epithelium, induced by smoking in this study, may be attributed to the effects of both smoking-derived TCDD and nicotine. Further studies are warranted to investigate the relationship between TCDD and miR-210-5p expression in mammary epithelia.

STAT5 plays a crucial role in the synthesis of various milk proteins, including α-casein, adiponectin, and β-casein [9]. Among these, β-casein serves as an important marker for mammary epithelial cell differentiation, which is essential for milk production [10]. In this study, we focused solely on evaluating the impact of nicotine on β-casein expression. Further studies should explore the effects of nicotine and smoking on the synthesis of other milk proteins, such as α-casein and adiponectin, within the mammary epithelium.

Maternal exposure to nicotine during the perinatal period causes aberrant fetal brain development, leading to increased vulnerability to brain hypo-ischemic encephalopathy in neonatal rats [35,36]. Wang et al. showed that nicotine administration to pregnant rats increased miR-210 expression in the brain and causes the onset of brain hypoxic-ischemic injury in neonatal rats. This implies that miR-210 plays an important role in aberrant brain development [37]. Ren et al. reported that the levels of miR-210-5p expression in the hippocampal tissues of rats with vascular dementia are significantly higher than those of neurotypical rats [38]. The same study showed that miR-210-5p is associated with early vascular dementia via a decrease in synapse number and inhibition of the synaptosomal-associated protein 25 (Snap25) in hippocampal neurons [38]. Breast milk miRNAs are enclosed by exosomes and are transferred to the infant's circulation without disintegration in the stomach [39]. Infants who consume breast milk containing miR-210-5p, increased by smoking-derived nicotine, may have a higher risk of brain-related diseases, including early vascular dementia. Further studies are required to investigate the influence of increased miR-210 expression in breast milk on infant brain development.

Our study had some limitations. First, we did not assess the specific level to which CS exposure in mice relates to smoking in humans. In this study, the miR-210-5p levels in the lungs of mice were used to indicate smoking. Future studies should investigate the association between the levels of CS exposure in the mouse model and smoking levels in humans using inflammatory biomarkers, such as interleukin-6 and interleukin-8, and not solely miR-210 levels. Second, the mechanisms behind the nicotine-induced increase in miR-210 expression were unclear. Further studies will be conducted to identify the nAChRs involved in miR-210 expression and investigate the nAChRs expression pattern in mammary epithelial cells of lactating women.

In conclusion, we found that maternal smoking during lactation decreased β-casein expression in the mammary epithelium, resulting in a reduction of the β-casein concentration in the milk of mice. Additionally, our results showed that smoking induced miR-210-5p expression in the mammary epithelium, and this induction decreased the protein levels of pSTAT5 and β-casein via inhibition of the STAT5 protein levels. This study also indicated that nicotine is specifically involved in the smoking-induced decrease of β-casein concentrations and the increase in miR-210-5p expression observed in milk. Our results demonstrate the detrimental effects that maternal smoking has on breast milk. These findings will better inform healthcare professionals and general citizens.

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Data availability

Data will be made available on request.

CRediT authorship contribution statement

Takeshi Chiba: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Akira Takaguri:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Toshiyasu Mikuma:** Investigation, Data curation. **Toshimi Kimura:** Investigation. **Tomoji Maeda:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Takeshi Chiba reports financial support was provided by Japanese Society for the Promotion of Science. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101773.

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