The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

*Toxicomics Report*

# Indeterminate alteration of TRPM8 and p21/Cip1 levels in normal human keratinocytes by incubating with medium including substances released by squamous carcinoma cells

**Tomofumi Fujino and Saki Ohkawa**

*Department of Hygiene and Health Sciences, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji 192-0392, Japan*

(Received January 26, 2024; Accepted February 3, 2024)

**ABSTRACT** — TRPM8, sensor of cold temperatures, regulates epidermal cell proliferation through CDK inhibitor p21/Cip1 and downregulation of TRPM8 causes p21/Cip1 decrease, associated with carcinogenesis. Given that lipids-activated nuclear receptor PPAR gamma negatively regulates the expression of TRPM8 in normal epidermal cells and that carcinoma cells generally secrete exosomes including various lipids, we examined whether TRPM8 and p21/Cip1 expressions in normal human keratinocytes are altered by incubating with medium including substances secreted by squamous carcinoma cells. TRPM8 and p21/Cip1 expressions in normal human keratinocytes HaCaT cells are altered by incubating with medium including substances secreted by squamous carcinoma SAS cells ("SAS medium"), however, the alteration of TRPM8 and p21/Cip1 expressions is indeterminate. In some case, "SAS medium" increased p21/Cip1 in TRPM8-independent manner whereas it decreased p21/Cip1 through both of TRPM8-dependent and independent pathway in other case. We also obtained the data showing that "SAS medium"-induced TRPM8 increase did not result in p21/Cip1 increase, probably from offset by TRPM8-independent downregulation of p21/Cip1. In all cases PPAR gamma level was not altered and "SAS medium" decreased TRPM8 level of HaCaT cells even when PPAR gamma was knocked down, indicating PPAR gamma-independent regulation of TRPM8 by "SAS medium". These results suggest that squamous carcinoma cells secrete various substances which increase and decrease p21/Cip1 level in nearby normal epithelial cells. Ratio of amounts of substances secreted by squamous carcinoma cells may vary depending on the cell condition and increasing ratio of substances which downregulates p21/Cip1 expression results in increased risk of carcinogenesis.

**Key words:** TRPM8, p21/Cip1, Normal human keratinocytes, Squamous carcinoma cells

## INTRODUCTION

TRPM8 is a calcium-permeable, non-selective cation channel of the transient receptor potential (TRP) superfamily, required for the transduction of moderate cold temperatures (Peier *et al.*, 2002). TRPM8 is highly expressed in several carcinoma cells and regulates its proliferation (Valero *et al.*, 2012; Yee *et al.*, 2010; Hantute-Ghesquier *et al.*, 2018; Chen *et al.*, 2014; Guo *et al.*, 2012). We previously found that the CDK inhibitor p21/Cip1 is a key factor involved in the regulation of TRPM8-mediated proliferation of epidermal

Correspondence: Tomofumi Fujino (E-mail: tfujino@toyaku.ac.jp)

cells, which are directly affected by cold temperatures (Fujino, 2022). p21/Cip1 causes G1 arrest resulting in decreased DNA synthesis while downregulation of p21/Cip1 is associated with carcinogenesis (Paramio *et al.,* 2001; Jackson *et al.,* 2002). Thus, TRPM8 downregulation may increase the risk of carcinogenesis attributed to a decrease in p21/Cip1 and stimulated proliferation of carcinoma cells. Reccently, we found that PPAR gamma, a nuclear receptor activated by various kinds of lipids, negatively regulates the expression of TRPM8 in normal epidermal cells but mutually regulate their expressions with TRPM8 by feed-back loop regulation in squamous carcinoma cells (Fujino *et al.*, 2023). Given that carcinoma cells generally secrete exosomes including various lipids (Thery *et al.*, 2002), we examined whether TRPM8 and p21/Cip1 expressions in normal human keratinocytes are altered by incubating with medium including substances secreted by squamous carcinoma cells.

## MATERIALS AND METHODS

### Materials

siRNA against PPAR gamma was purchased from Santa Cruz Biotechnology (Dallas, USA).

### Cell culture

HaCaT cells derived from normal human keratinocytes (CLS Cell Lines Service, 300493) (Boukamp *et al*., 1988) and squamous carcinoma SAS cells (Takahashi *et al*., 1989) were maintained in Dulbecco's modified eagle medium containing 10% fetal calf serum, 50 units/mL penicillin G sodium salt, and 50 μg/mL streptomycin sulfate and cultured in a humidified atmosphere of 8.5% $CO_2$ at 37°C.

### RNA interference experiments

To knockdown endogenous PPAR gamma, cells were seeded on 60-mm dishes at a density of $2.0 \times 10^5$ cells per dish and transfected with siRNA against PPAR gamma (10 nM) using HiPerfect Transfection Reagent (Qiagen) according to the manufacturer's instructions. After incubating for 24 hr, total RNA was extracted for real-time polymerase chain reaction (PCR). In RNA interference experiments, "Nonsilencing Control" siRNA (#1022076) from Qiagen was used as a control.

### Quantification of mRNA

Quantification of mRNA was performed using real-time PCR. Briefly, 4 μg of total RNA was reverse-transcribed using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The resultant cDNA was subjected to real-time PCR analysis using a TaqMan Gene Expression Assay kit (Applied Biosystems, Tokyo, Japan). mRNA levels were determined using TaqMan assay mixtures for TRPM8 (Hs00368574), p21/Cip1 (Hs01121172), PPAR gamma (Hs01115513), and β-actin (4310881E). Amplification and quantification were performed using the StepOne Real-Time PCR System (Applied Biosystems). mRNA levels were normalized to those of β-actin as an internal control.

### Statistical analysis

Data are presented as the mean ± S.E.M. of three experiments performed in triplicate and were analyzed using Two-way ANOVA with Bonferroni post-hoc test.

## RESULTS AND DISCUSSION

We examined whether TRPM8 and p21/Cip1 expressions in normal human keratinocytes are altered by incubating with medium including substances secreted by squamous carcinoma cells, like as Fig. 1A. As shown in Fig. 1B-E, TRPM8 and p21/Cip1 expressions in normal human keratinocytes were indeterminately altered by incubating with medium including substances secreted by squamous carcinoma SAS cells (we call "SAS medium" in this paper). In experiments 1 (Fig. 1B), p21/Cip1 level was increased by "SAS medium" without changing of TRPM8 level. In experiment 2 (Fig. 1C), both of TRPM8 and p21/Cip1 levels were decreased. In experiment 3 (Fig. 1D), p21/Cip1 level was decreased without changing of TRPM8 level. In experiment 4 (Fig. 1E), TRPM8 level was increased without changing of p21/Cip1 level. p21/Cip1 increase without changing of TRPM8 level in experiments 1 indicate that the p21/Cip1 increase was TRPM8 independent. Although p21/Cip1 was similarly decreased in experiment 2 and 3, only p21/Cip1 decrease in experiment 2 accompanied TRPM8 decrease, indicating that the p21/Cip1 decrease was caused by TRPM8 decrease while p21/Cip1 decrease in experiment 3 was TRPM8 independent. Although TRPM8 increase in experiment 4 probably stimulated p21/Cip1 expression, p21/Cip1 increase by TRPM8 was supposed to be offset by TRPM8-independent downregulation of p21/Cip1. In contrast to incubation of HaCaT cells with "SAS medium", TRPM8 and p21/Cip1 levels in SAS cells were not altered by incubating with the medium including substances secreted by HaCaT cells (we call "HaCaT medium" in this paper), indicating that HaCaT cells did not secrete substances which affect TRPM8 and p21/Cip1 expressions in SAS cells. Both cells probably do not secrete substances which affect TRPM8 and p21/

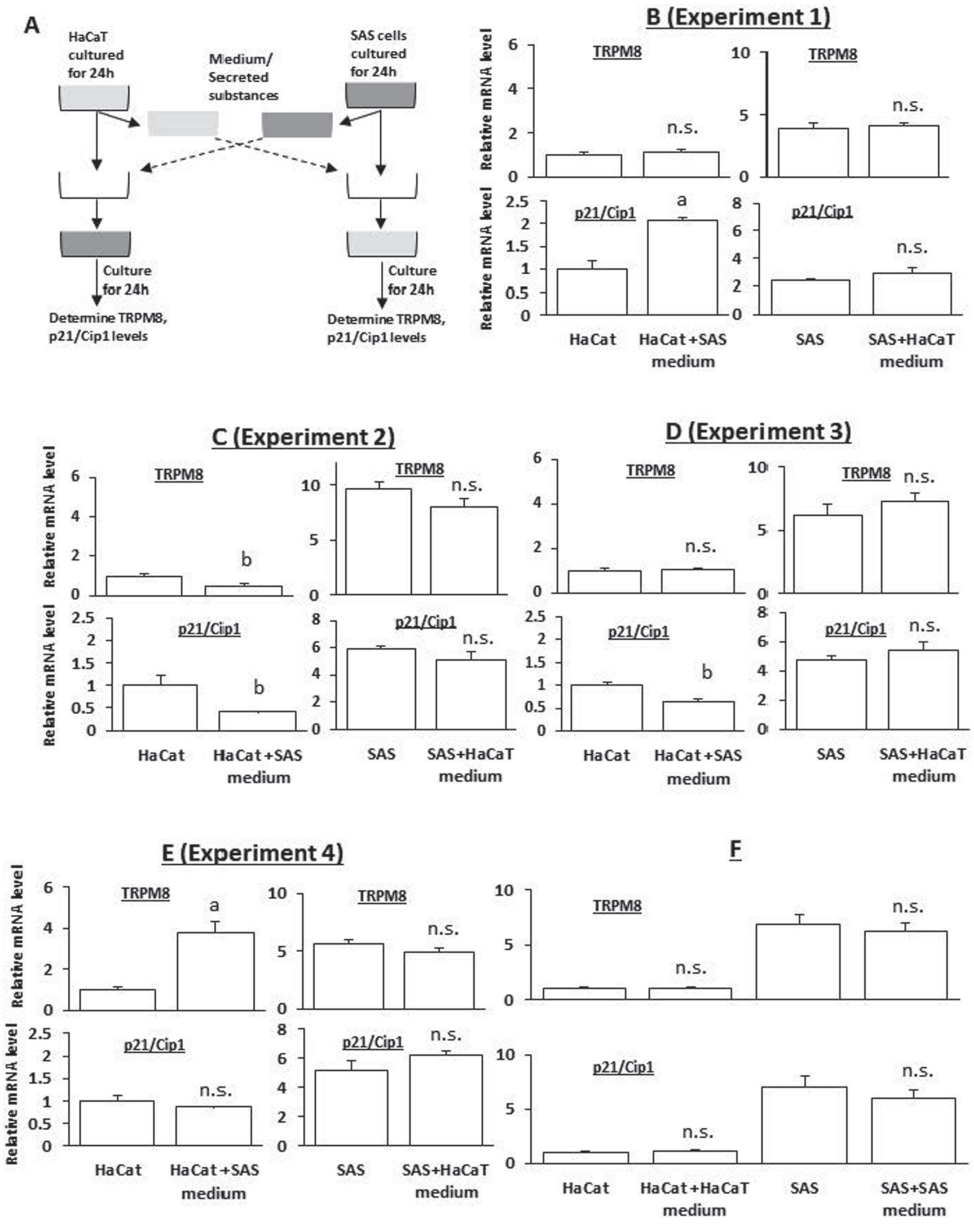


**Fig. 1**. TRPM8 and p21/Cip1 expressions in normal human keratinocytes are indeterminately altered by incubating with medium including substances secreted by squamous carcinoma cells ("SAS medium") while that in squamous carcinoma cells are not affected by medium including substances secreted by normal keratinocytes ("HaCaT medium"). A: scheme of experiments1-4. B-E: HaCaT and SAS cells seeded at $2.0 \times 10^5$ cells/60-mm dish were cultured for 24 hr. Then medium of HaCaT and SAS cells were exchanged each other and incubated for 24 hr. Total RNA was quantified to determine TRPM8 and p21/Cip1 mRNA levels as described in the **Materials and Methods**. Four experiments were performed in triplicate. Data were analyzed using Two-way ANOVA with Bonferroni post-hoc test and are presented as the mean ± S.E.M. of each experiment performed in triplicate. F: HaCaT and SAS cells seeded duplicate at $2.0 \times 10^5$ cells/60-mm dish were cultured for 24 hr. Then medium of two dishes of HaCaT cells were exchanged each other and incubated for 24 hr. For SAS cells, similar experiment was performed. Total RNA was quantified to determine TRPM8 and p21/Cip1 mRNA levels as described in the **Materials and Methods.** Data were analyzed using Two-way ANOVA with Bonferroni post-hoc test and are presented as the mean ± S.E.M. of three experiments performed in triplicate. n.s.: not significant. a: significant compared to normally cultured HaCaT cells; $P < 0.01$. b: significant compared to normally cultured HaCaT cells; $P < 0.05$.

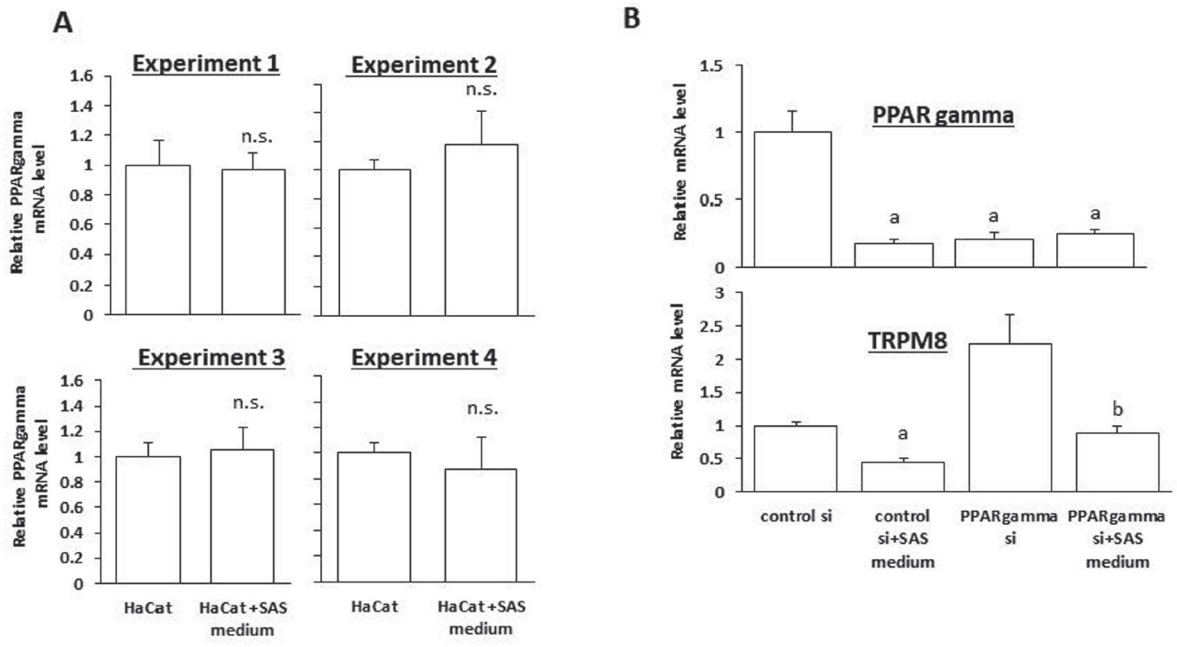


**Fig. 2**. "SAS medium" does not alter PPAR gamma level and decreased TRPM8 level even when PPAR gamma is knocked down in normal keratinocytes. A: total RNA obtained in experiments 1-4 (Fig. 1B-E) was quantified to determine PPAR gamma mRNA level as described in the **Materials and Methods.** B: HaCaT seeded at $2.0 \times 10^5$ cells/60-mm dish were transfected with siRNA against PPAR gamma, or control siRNA. SAS cells was seeded at $2.0 \times 10^5$ cells/60-mm dish. After 24 hr, "SAS medium" was added to siRNA-transfected cells, followed by incubation for 24 hr. Total RNA was quantified to determine TRPM8 and PPAR gamma mRNA levels as described in the **Materials and Methods.** Data were analyzed using Two-way ANOVA with Bonferroni post-hoc test and are presented as the mean ± S.E.M. of each experiment performed in triplicate. n.s.: not significant. a: significant compared to "control si"; $P < 0.01$. b: significant compared to "PPAR gamma si"; $P < 0.05$.

Cip1 expressions of themselves, as shown in Fig. 1F. In HaCaT cells, TRPM8 expression is negatively regulated by PPAR gamma as we previously reported (Fujino *et al*, 2023), however, alteration of TRPM8 and p21/Cip1 in HaCaT cells by incubating with the "SAS medium" was not associated with changing of PPAR gamma level (Fig. 2A). Moreover, "SAS medium" similarly downregulated TRPM8 expression even when PPAR gamma was knocked down (Fig. 2B), indicating that SAS cells secrete substances which downregulate TRPM8 expression in PPAR gamma-independent manner.

In the present study, we revealed that TRPM8 and p21/Cip1 expressions in normal human keratinocytes HaCaT cells are altered by incubating with medium including substances secreted by squamous carcinoma SAS cells ("SAS medium"), indicating that squamous carcinoma SAS cells secrete substances which affect TRPM8 and p21/Cip1 expressions in normal human keratinocytes. Normal human keratinocytes do not affect TRPM8 and p21/Cip1 expressions of squamous carcinoma cells. It was very surprising and interesting that "SAS medium" indeterminately affected TRPM8 and p21/Cip1 expressions in normal human keratinocytes. Squamous carcinoma cells secrete substance upregulating p21/Cip1 expression without affecting TRPM8 level, substance downregulating p21/Cip1 expression with or without affecting TRPM8 level, substance upregulating TRPM8. Relative ratio of secreted amount of those substances probably varies depending on cell condition. Thus, when squamous carcinoma cells secrete large amounts of substances which decrease p21/Cip1 level, it increases the risk of carcinogenesis in nearby normal epithelial cells. Elucidation of the correlation between the carcinoma cell conditions and ratio of secretion of substances affecting TRPM8 and p21/Cip1 expressions greatly contribute the cancer therapy.

## ACKNOWLEDGMENTS

We thank Atsushi Shoji, Kazuhiro Morioka, Yoshinori Inoue, Makio Hayakawa, and Toshiyuki Oshima for their encouragement. This work was sup-

ported in part by a grant from the Japan Private School Promotion Foundation.

**Conflict of interest----** The authors declare that there is no conflict of interest.

## REFERENCES

Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A. and Fusenig, N.E. (1988): Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol., **106**, 761-771.

Chen, J., Luan, Y., Yu, R., Zhang, Z., Zhang, J. and Wang, W. (2014): Transient receptor potential (TRP) channels, promising potential diagnostic and therapeutic tools for cancer. Biosci. Trends, **8**, 1-10.

Fujino, T. (2022): Transient Receptor Potential Melastatin 8, a sensor of cold temperatures mediates expression of cyclin-dependent kinase inhibitor, p21/Cip1, a regulator of epidermal cell proliferation. J. Toxicol. Sci., **47**, 117-123.

Fujino, T., Nakamura, M. and Ohkawa, S. (2023): PPAR gamma negatively regulates the expression of TRPM8 in normal epidermal cells but mutually regulate their expressions with TRPM8 by feed-back loop regulation in squamous carcinoma cells. Fundam. Toxicol. Sci., **10**, 1-6.

Guo, H., Carlson, J.A. and Slominski, A. (2012): Role of TRPM in melanocytes and melanoma. Exp. Dermatol., **21**, 650-654.

Hantute-Ghesquier, A., Haustrate, A., Prevarskaya, N. and Lehen'kyi, V. (2018): TRPM Family Channels in Cancer. Pharmaceuticals (Basel), **11**, 58.

Jackson, R.J., Adnane, J., Coppola, D., Cantor, A., Sebti, S.M. and Pledger, W.J. (2002): Loss of the cell cycle inhibitors p21(Cip1) and p27(Kip1) enhances tumorigenesis in knockout mouse models. Oncogene, **21**, 8486-8497.

Paramio, J.M., Segrelles, C., Ruiz, S., Martin-Caballero, J., Page, A., Martinez, J., Serrano, M. and Jorcano, J.L. (2001): The ink4a/arf tumor suppressors cooperate with p21cip1/waf in the processes of mouse epidermal differentiation, senescence, and carcinogenesis. J. Biol. Chem., **276**, 44203-44211.

Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S. and Patapoutian, A. (2002): A TRP channel that senses cold stimuli and menthol. Cell, **108**, 705-715.

Takahashi, K., Kanazawa, H., Akiyama, Y., Tazaki, S., Takahara, M., Muto, T., Tanzawa, H. and Sato, K. (1989): Establishment and characterization of a cell line (SAS) from poorly differentiated human squamous cell carcinoma of the tongue. J. Jpn. Stomatol. Soc., **38**, 20-28.

Thery, C., Zitvogel, L. and Amigorena, S. (2002): Exosomes: composition, biogenesis and function. Nat. Rev. Immunol., **2**, 569-579.

Valero, M.L., Mello de Queiroz, F., Stühmer, W., Viana, F. and Pardo, L.A. (2012): TRPM8 ion channels differentially modulate proliferation and cell cycle distribution of normal and cancer prostate cells. PLoS One, **7**, e51825.

Yee, N.S., Zhou, W. and Lee, M. (2010): Transient receptor potential channel TRPM8 is over-expressed and required for cellular proliferation in pancreatic adenocarcinoma. Cancer Lett., **297**, 49-55.