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Toxicomics Report

Increased expression of TRPM8 and p21/Cip1 in epithelial carcinoma cells in confluent conditions

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ABSTRACT — Based on that TRPM8 is a key factor of normal cell-carcinoma cell interaction and there is a tendency for proliferation of some cell types that are repressed in a high-density state rather than in a low-density state, we attempted to clarify the relationship between direct cell-cell interactions and expression of TRPM8, repressor of carcinoma cell proliferation. We examined TRPM8 and p21/Cip1 levels in normal and carcinoma epithelial cells at low and high cell density (confluent). TRPM8 and p21/Cip1 levels in confluent carcinoma cells markedly increased as compared with that of carcinoma cells in low density and normal cells in low density and confluent. When carcinoma cells cultured to confluent were re-seeded at low density, TRPM8 and p21/Cip1 levels decreased, indicating that the direct cell-cell interaction may regulate the expression of TRPM8 and p21/Cip1 in carcinoma cells. Totally, TRPM8 functions as repressor of cell proliferation in the condition which enables direct interaction between carcinoma cells occurs.

Key words: TRPM8, p21/Cip1, Direct interaction between carcinoma cells

INTRODUCTION

TRPM8, a member of the TRP family that is widely involved in various cellular functions, is an ion channel activated by cold and cold pain (Peier *et al*., 2002). TRPM8 is also known to increase the expression of cyclin-dependent kinase inhibitor p21/Cip1 and inhibit proliferation (Fujino, 2022). Recently, we have reported that TRPM8 is a key factor of normal cell-carcinoma cell interaction. Epithelial carcinoma cells have been shown to release both factors that increase and decrease TRPM8 and p21/Cip1 levels in normal cells (Fujino and Ohkawa, 2024). TRPM8 level of the carcinoma cell itself is not affected by substances released from carcinoma cell, and the normal cell does not release the factor that affects the TRPM8 level of normal cells and cancer cells. On the other hand, our preliminary data shows that in cell culture of some cell types, there is a tendency for proliferation to be repressed in a high-density state rather than in a low-density state. Therefore, in order to clarify the relationship between direct cell-cell interactions and expression of TRPM8, repressor of carcinoma cell proliferation, we examined TRPM8 and p21/Cip1 levels in normal and carcinoma epithelial cells at low and high cell density.

MATERIALS AND METHODS

Cell culture

HaCaT cells derived from normal human keratinocytes (CLS Cell Lines Service, 300493) (Boukamp *et al*., 1988), squamous carcinoma cell line SAS (Takahashi *et al*., 1989), human lung carcinoma cell line A549 were maintained in Dulbecco's modified eagle medium containing 10% fetal calf serum, 50 units/mL penicillin G

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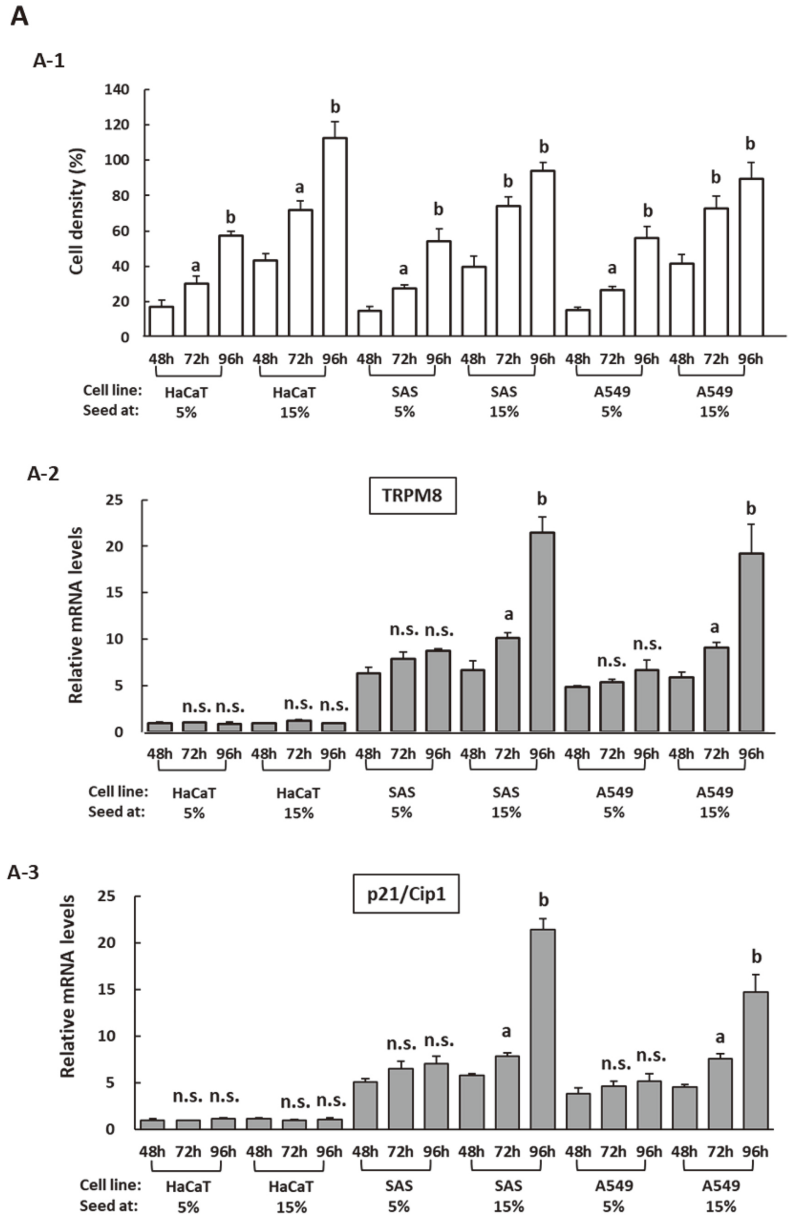

Fig. 1. Remarkable increase in TRPM8 and p21/Cip1 in confluent carcinoma cells.
Data was analyzed using Two-way ANOVA with Bonferroni post-hoc test and are presented as the mean ± S.E.M. of three experiments performed in triplicate.
A: HaCaT, SAS, A549 cells seeded at 5.0×10^4 or 1.5×10^5 cells/60-mm dish were incubated for 48-96 hr. Cell number was counted and cell density was calculated using cell number of confluent cells as a standard (A-1). Total RNA was quantified to determine TRPM8, p21/Cip1 mRNA levels (A-2, 3) as described in the **Materials and Methods**.
n.s.: not significant
a: significant compared to "cells incubated for 48 hr"; $P<0.05$
b: significant compared to "cells incubated for 48 hr"; $P<0.01$
B: SAS and A549 cells seeded at 20% confluent were incubated to confluent. The confluent cells were collected and re-seeded at 5% confluent. After 24 hr, total RNA was quantified to determine TRPM8 and p21/Cip1 mRNA levels as described in the **Materials and Methods**. b: significant compared to "SAS or A549 confluent"; $P<0.01$
C: SAS and A549 cells seeded at 20% confluent were incubated to confluent. Total RNA was quantified to determine PPAR-gamma mRNA levels as described in the **Materials and Methods**.
n.s.: not significant to "SAS or A549 20%"

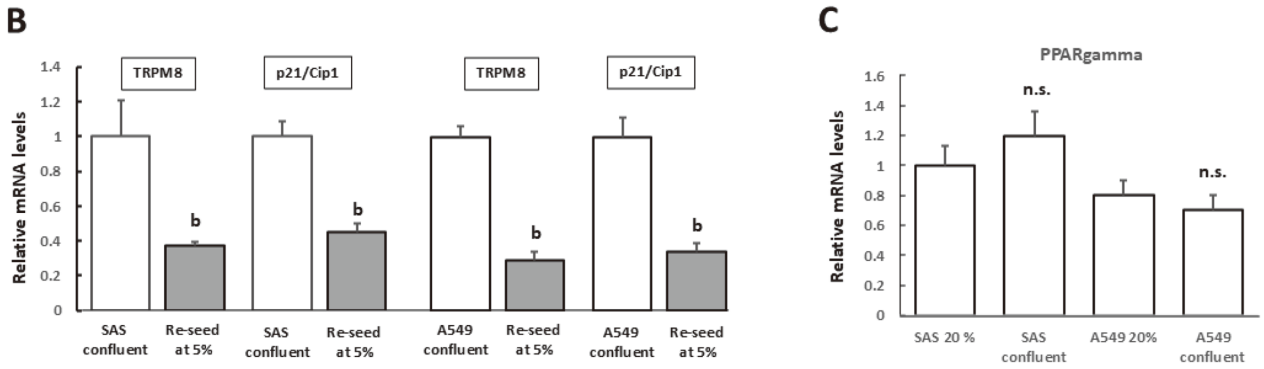

Fig. 1. (Continued).

sodium salt, and 50 μg/mL streptomycin sulfate and cultured in a humidified atmosphere of 8.5% CO_2 at 37°C.

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

Human normal epithelial cells HaCaT and squamous carcinoma cell line SAS were seeded at different cell densities (5×10^4 cells, 1.5×10^5 cells/60 mm dish) and collected after 48, 72, 96 hr to measure cell density, TRPM8 and p21/ Cip1 levels. SAS cells seeded at low density grew to a density of 54% confluent after 96 hr, but there was no significant change in TRPM8 and p21/Cip1 levels as compared to cells incubated 48 hr ("SAS 5%" in Fig. 1A). SAS cells seeded at high density grew to 94% confluent after 96 hr, and TRPM8 and p21/Cip1 levels were remarkably elevated as compared to cells incubated 48 hr ("SAS 15%" in Fig. 1A). On the other hand, HaCaT cells seeded at high density grew to the same extent as in SAS cells, but TRPM8 and p21/Cip1 levels did not change ("HaCaT 15%" in Fig. 1A). In human lung cancer cell A549, as in SAS cells, TRPM8 and p21/Cip1 levels markedly increased when the cell density reached nearly confluent ("A549 15%" in Fig. 1A). When SAS and A549 cells cultured to confluent were re-seeded at low density, TRPM8 and p21/Cip1 levels decreased (Fig. 1B), indicating that the direct cell-cell interaction may regulate the expression of TRPM8 and p21/Cip1 in cancer cells. Based on our previous report that PPARgamma is a regulator of TRPM8 expression in cancer cells (Fujino *et al.,* 2023), we compared PPARgamma levels of 20% confluent and confluent carcinoma cells, but there was no difference in PPARgamma levels between these cells (Fig. 1C), indicating that the TRPM8 and p21/Cip1 increase in confluent carcinoma cells is not related to PPARgamma.

The correlation between TRP family factors and tight junctions, multiprotein junctional complexes between epithelial cells, has been reported, such as TRPV4 regulates tight junctions between cells (Islam *et al.,* 2020), although the role of TRPM8 in tight junction is unknown. In addition, tight junctions are known to contribute to promoting cell proliferation and endothelial-mesenchymal transition (EMT) (Díaz-Coránguez *et al.,* 2019). Interestingly, the present study shows that TRP family TRPM8 and p21/Cip1 increase, probably resulting in inhibited cell proliferation, in confluent epithelial carcinoma cells where the cell-cell interaction occurs. p21/Cip1

has been also reported to suppress Endothelial-Mesenchymal Transition (EMT), initial stage of cancer metastasis (Liu *et al.,* 2009). Thus, the risk of proliferation and metastasis may be reduced if we can make the condition that carcinoma cells locally dense, elevating TRPM8 and p21/Cip1 expression. Moreover, if the resection of densely packed carcinoma cells is insufficient, loss of cell-cell interaction in remaining cells may increase malignancy. Further research is needed to establish a novel treatment of cancer.

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

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