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Ribosome induces transdifferentiation of A549 and H-111-TC cancer cell lines

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ABSTRACT

Previously we reported that, lactic acid bacteria (LAB) can induce human dermal fibroblast (HDF) cells to form multipotent cell clusters which are able to transdifferentiate into three germ layer derived cell lineages. Later on, we confirmed that ribosome is responsible for the LAB-induced transdifferentiation and ribosomes from diverse organisms can mimic the LAB effect on HDF cells. In our present study we have shown that, upon incorporation of ribosomes, non-small cell lung cancer cell line A549 and gastric tubular adenocarcinoma cell line H-111-TC are transformed into spheroid like morphology those can be transdifferentiated into adipocytes and osteoblast. Our qPCR analysis has revealed that, during the formation of ribosome induced cancer cell spheroids, the expression of the cancer cell associated markers and cell cycle/proliferation markers were altered at different time point. Through our investigation, here we report a novel and a non-invasive approach for cancer cell reprogramming by incorporating ribosomes.

1. Introduction

Cancer progression, reprogramming of the terminally differentiated cells, dedifferentiation and transdifferentiation are some of the phenomena directed by numerous extrinsic/intrinsic stimuli, epigenetic factors and inherent genetic predispositions [1–3]. These are deviations of the cellular plasticity which we observe in a unidirectional manner towards the normal development and physiological homeostasis maintenance [4]. The concept of the cell reprograming is more than fifty years old that followed later with another two remarkable discoveries: Dolly, the cloned sheep and the induced pluripotent stem cell (iPS), triggered by famous Yamanaka's factors (*Oct4, Sox2, c-Myc and Klf4*) [5–7]. However, perception of cancer stem cell (CSC) and its plasticity have encouraged to utilize cell reprograming techniques like somatic cell nuclear transfer (SCNT), iPS approach and direct reprograming (transdifferentiation) to reverse more invasive malignant state into

benign state [8,9]. Among these techniques transdifferentiation is promising over others in respect with bypassing the malignant formation, though its efficiency needs to be optimized further with better understanding of the mechanism underlying it [10]. A minor fraction of CSCs, which can be seen across the small cell lung carcinomas, breast cancers, pancreatic carcinomas and hepatocellular carcinomas [11–14], is observed to transform into tumorigenic and non-tumorigenic cells that creates the heterogeneity of the tumor microenvironment. However, we do not know much about the tumor heterogeneity and it remains to be one of the elusive factors in the way to measure the efficiency of cancer cell reprogramming.

In recent times, successful cancer cell reprogramming has been achieved through introducing lineage specific transcription factors, small molecules, miRNAs and even with exosomes mediated reprogramming [8]. Previously our lab reported that, lactic acid bacteria (LAB) is able to trigger cellular reprograming of the human dermal fibroblasts

(HDF) that differentiated into three germ layer cells [15]. Another group later reported Leprocy Bacilli induced adult schwann cell reprogramming which allowed differentiation directly into mesenchymal, skeletal and smooth muscle tissues [16]. In further studies, we found that, it was the ribosome fraction within the LAB that was directing the reprograming phenomenon [17]. The embryoid like cell cluster, formed by the incorporation of ribosome into the trypsinized HDF cells, transdifferentiated into three germ layer cells in the induction medium, expressed pluripotency markers and showed reduced cell proliferation [17]. We know well about the ribosome as a protein manufacturing organelle in the cell. But it possesses an interesting attribute, which is called "moonlighting", in performing other functions apart from his original function like regulating cancer progression, immune-modulation and playing roles even in the developmental processes [18,19]. Here we report that, incorporation of ribosome transformed non-small cell lung cancer cell line A549 and gastric tubular adenocarcinoma cell line H-111-TC into spheroid like structures, those were able to transdifferentiate directly into adipocytes and osteoblast in the induction medium. Our qPCR analysis showed that, expression of several cancer cell associated, and cell cycle/proliferation markers were altered in the ribosome induced cancer cell spheroids (RICCSs) at different time point. Our *in vivo* tumor formation assay showed minute reflection of our *in vitro* results. Overall, in our present study, we attempted to introduce a novel approach in direct reprogramming of cancer cells by incorporating ribosomes.

2. Material and methods

2.1. Culture of cancer cells and bacteria

A549 and H-111-TC cancer cell lines used in this study were obtained from Riken BRC Cell Engineering Division – Cell Bank – (https://cell.brc.riken.jp/en/). *Escherichia coli* JE28 bacterial strain was obtained from Uppsala University, Sweden, Department of Cell and Molecular Biology (https://www.icm.uu.se/molecular-biology/sanyal-lab/je-28-request/). The guidelines of the Committee on Animal Research at the Kumamoto University and Institutional Committee of Laboratory Animal Experimentation of the RIKEN Center for Developmental Biology were followed for performing cell culture experiments. Dulbecco Modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) were used for control cancer cell culture and were maintained in the

incubator with the atmospheric condition of 37 °C and 5% CO_2.

2.2. Animal

For tumor formation assay Rag-2/Jak3 double deficient mice with BALB/c genetic background were used [20]. All experiments on mouse were performed in accordance with the guideline of the Committee on Animal Research at University of Kumamoto. All animal experiments were conducted according to the guidelines of Animal Research: *In Vivo* Experiment Report (ARRIVE) [21].

2.3. Isolation and purification of ribosome

The detailed protocol for isolation and purification of ribosome from *E. coli* JE28 was previously described [17,22].

2.4. Formation and transdifferentiation of RICCS

The confluent (80–95%) cancer cells culture were trypsinized and 1 $\times$ 10^5 cells were suspended in 500 μl PluriSTEM™ Human ES/iPS cell medium (Cat. SCM130, Merck) per well of a 2 cm culture dish after mixing with appropriate amount of purified ribosome. A control plate was prepared for each sample plate. Day after the incorporation of ribosome another 500 μl PluriSTEM™ Human ES/iPS cell medium was added to each well. Half of the medium was replaced in every two/three days interval. One set of cells was cultured up to one week (D7) and another set up to two weeks (D14). The cDNA was prepared from each set of control wells as well as from RICCS sample wells. For transdifferentiation, one or two RICCSs were collected from D14 culture and suspended in the specific differentiation induction medium. Adipocyte Differentiation Basal medium StemPro® (gibco, Cat No. A10410-01), Osteoblast Differentiation Basal Medium StemPro® (gibco, Cat No. A10069-01), Chondrocyte Differentiation Basal Medium StemPro® (gibco, Cat No. A10069-01) were used to differentiate the RICCS into adipocytes, osteoblast and chondrocytes respectively. The differentiated adipocytes were stained with Oil red O (SIGMA-ALDRICH, Lot # SLBP5248V), Osteoblasts with Alizarin Red S (SIGMA-ALDRICH, A5533-25G) and Chondrocytes with Alician Blue Stain Solution (nacalai tesque, Lot No. L1E7365) after two weeks of culture in the differentiation medium.

2.5. qPCR analysis

The RNA from control culture (CC) and two sets of RICC culture (D7 and D14) was isolated and purified by using the RNeasy® Mini Kit (QIAGEN, Cat No. 74106). cDNA was prepared from these purified RNAs using Superscript™ II RT (200 U) enzyme kit (Invitrogen Cat No. 18064022). For qPCR analysis Luna® Universal qPCR Master Mix (NEW ENGLAND BioLabs® Inc. Cat No. M3003E) was used. We selected Epidermal Growth Factor Receptor (EGFR) and C-X-C Motif Chemokine Receptor 4 (CXCR4) as A549 associated markers [23,24]. But we could not find any suitable marker H-111-TC, therefore we continued with A549 in this study. We also investigated cell cycle/proliferation markers Ki67 and Cycline D1 (CycD1) [25,26]. Gene expression of these markers were normalized against Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) and β-Actin housekeeping genes. The following primer sets were used: EGFR – Forward: 5′-GCCACAGGCCAGGTCTGCCAT-3′, Reverse: 5′-CCGGCGTCTGCGTACTTCCAG-3′, CXCR4 – Forward: 5′-CCGTGGC-AAACTGGTACTTT-3′, Reverse: 5′-GACGCCAACATAGACCACCT-3′, Ki67 – Forward: 5′-TCCTTTGGTGGGCACCTAAGACCTG-3′, Reverse: 5′-TGATGG-TTGAGGTCGTTCCTTGATG-3′, CycD1 – Forward: 5′-AGCTCCTGTGCTG-CGAAGTGGAAAC-3′, Reverse: 5′-AGTGTTCAATGAAATCGTGCGGGGT-3′, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) – Forward: 5′- GTC-AACGGATTTGGTCGTATT -3′ Reverse: 5′- ATCACTGCCACCCAGAAGACT -3′. β-Actin – Forward: 5′- AAGATCAAGATCATTGCTCCTC -3′, Reverse: 5′-GGGTGTAACGCAACTAAGTC -3′.
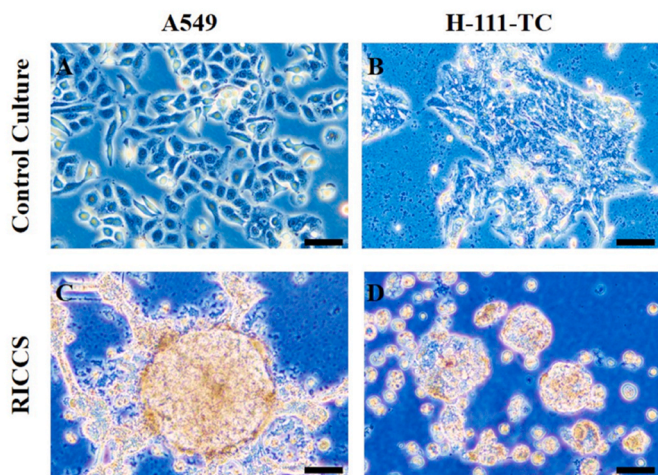

Fig. 1. Ribosome induced cancer cell spheroid (RICCS) formation. Control cultures of (A) non-small cell lung cancer cell line A549 and (B) gastric tubular adenocarcinoma cell line H-111-TC are shown in upper panel. After ribosome incorporation the corresponding cancer cell lines were cultured up to 14 days. RICCSs from (C) A549 and (D) H-111-TC are shown in lower panel. For each cancer cell line three independent experiments were performed. Bars: 70 μm.
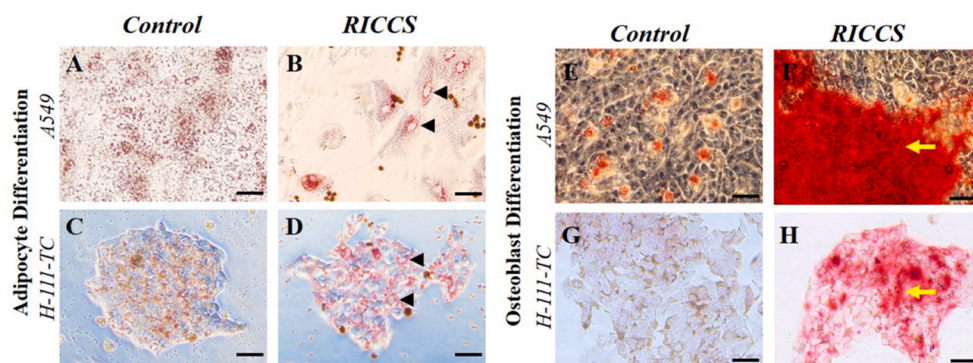
Fig. 2. Transdifferentiation of RICCSs into adipocyte and osteoblast. Control cells from (A) A549 and (C) H-111-TC and their corresponding (B and D) adipocyte transdifferentiation staining images are shown in the adjacent figures. Black arrowheads indicate adipocytes staining. Control cells (E and G) and their corresponding (F and H) osteoblast transdifferentiation are shown in the adjacent figures. Yellow long arrows indicate osteoblast staining. For each cancer cell line three independent experiments were performed. Bars: 70 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.6. In vivo tumor formation assay

Target sites of control mice group were injected subcutaneously (SC) (3×10^6 cells/sites) with A549 cells and the experimental group with A549 RICCS cells (trypsinized). After the tumors were visible on approximately day 17th (D-17), 1X phosphate buffer solution (PBS) was treated into the tumors of the control group and the experimental group was treated with purified ribosomes. Tumors were pre-treated briefly with the Trypsin-EDTA followed by washing with the 1X PBS of both group of mice. This procedure was continued on D-21, 24 and 28. On D-30 the mice were sacrificed, tumors were dissected and analyzed.

3. Results

3.1. Ribosome transforms cancer cells into spheroids

A549 and H-111-TC cancer cell lines started to show altered phenotypes within two to three days after the ribosome incorporation. On day seventh of the culture, spheroid formation appeared and on the day fourteenth almost all cells formed clear RICCSs. We observed a definite morphological alteration from control cancer cell culture of A549 (Fig. 1A) and H-111-TC (Fig. 1B) into spheroid like structure when ribosome was incorporated in the respective cell lines (Fig. 1C and D).

3.2. RICCS transdifferentiate into multilineage cells

After finding the result of spheroid formation from cancer cells, we investigated if RICCS could be transdifferentiated into three germ layer cell lineages like our previous study performed on HDF [17]. We picked up one or two of the spheroids and let them differentiate up to two weeks in the specific induction medium and afterwards we stained with linage

specific dyes to observe those differentiated cells. We observed that, in compared to controls of A549 (Fig. 2A) and H-111-TC (Fig. 2C) all spheroids from respective cancer cell lines were transdifferentiated into adipocytes (Fig. 2B and D). For osteoblast staining, all controls showed negative staining result (Fig. 2E and G) while their RICCS counterparts (Fig. 2F and H) showed very translucent positive staining result. We could not conclude on chondrocyte staining result as most of the cells of control and RICCS sample did not survive in the induction medium (data not shown).

3.3. Ribosome induction alters oncogenic and proliferative status

We next concentrated on investigating the status of cancer cell relevant and cell cycle/proliferation markers. We compared marker expression of control cells with D7 – RICCS and D14 – RICCS samples. Interestingly we found that, A549 cell line associated marker EGFR was upregulated significantly in D7 – RICCS sample compared to control, but it was significantly downregulated in D14 – RICCS sample compared to D7 – RICCS sample (Fig. 3A). Expression of CXCR4, another A549 specific marker, showed increasing tendency in D14 – RICCS sample (Fig. 3B). Cell proliferation marker Ki67 was gradually upregulated from D7 – RICCS to D14 – RICCS samples in compared to CC (Fig. 3C). While expression of cell cycle marker CycD1 was at first upregulated on D7 – RICCS sample but later it was decreased in D14 – RICCS sample compared to control (Fig. 3D).

3.4. In vivo tumor formation assay shows ribosome has mere effect on tumor growth

Next, we observed the effect of ribosome incorporation in *in vivo* set up. We used Rag-2/Jak3 double deficient mice with BALB/c genetic
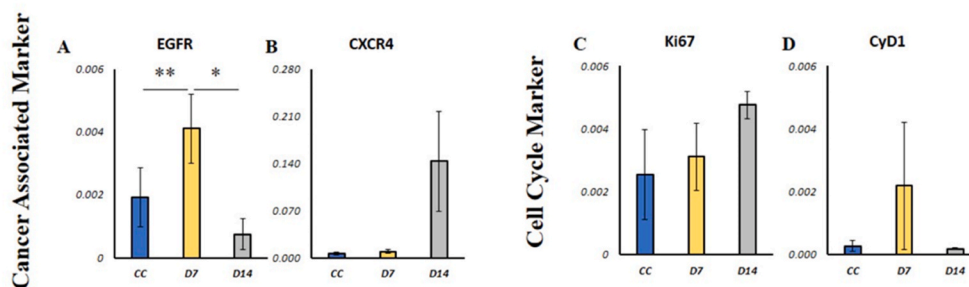

Fig. 3. Comparative qPCR analysis of A549 cancer cell line associated markers and cell cycle/proliferation markers between control culture (CC), D7 and D14 samples of RICCS. From the Left, analysis of (A) EGFR, (B) CXCR4, (C) Ki67 and (D) CycD1 are shown. All data are shown as the mean ± standard error of the mean (s.e.m.) from three independent experiments. Statistical analysis was performed using Student's *t*-test and significance was set as follows: *P < 0.05, **P < 0.01.
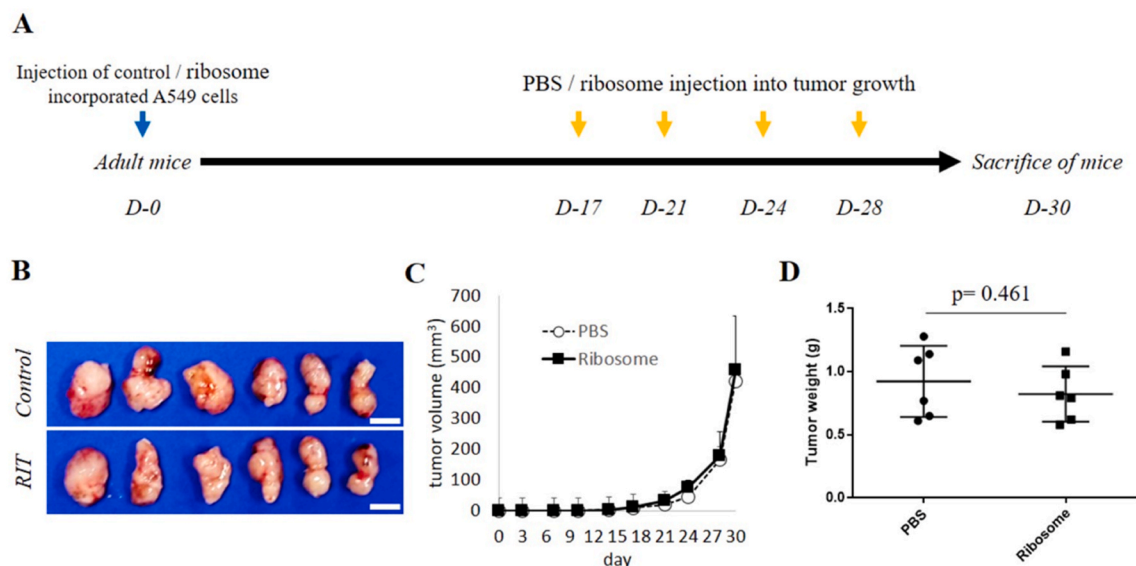
Fig. 4. *In vivo* tumor formation assay. (A) An outline of tumor formation assay is showing injection into adult mice with the control A549 cells and trypsinized cells from RICCS and subsequent steps. (B) Control tumors and ribosome incorporated tumors (RITs) are showing in the lower leftmost panel. Graphical representations are showing (C) changes of tumor volume with the progression of days and (D) comparison of tumor weight between control (PBS) and ribosome treated group. All data are shown as the mean $\pm$ standard error of the mean (s.e.m.) from three independent experiments. Statistical analysis was performed using Student's *t*-test and significance was set as follows: Bars: 1 cm.

background and among the two cell lines we chose A549 for performing *in vivo* tumor formation assay. We illustrated the tumor formation assay in Fig. 4A. Tumor size and volume were not found significantly different between the control group and those with the ribosome incorporated tumor (RIT) growth group within the experimental timeframe (Fig. 4B and C). But the overall weight of the tumors was reduced slightly following the ribosome incorporation (Fig. 4D).

4. Discussion

Alteration in ribosome biogenesis and stochastic functional abnormalities of ribosomal proteins within the cell are evidently associated with onset cancer development and its further progression [27]. But it is a novel and an intriguing approach to incorporate prokaryotic ribosome from outside of the cancer cell and thus observing the reprogramming phenomenon in our present investigation. Cell cluster morphology of embryonic stem cell (ES) and or iPS cells was reported previously [7], so we did observe when exogenous ribosome induced similar cancer cell spheroids in our study. Later we observed adipocyte and osteoblast transdifferentiation from RICCS that was the definitive proof of direct reprogramming from the cancer cells. But, adipocyte differentiation from A549 control cells was not well defined and we could not observe transdifferentiation into chondrocyte from any of these RICCS as they were unable to survive in the chondrocyte induction medium. However, from our study, it was difficult to explain how the ribosome is inducing the transdifferentiation of cancer cells, but it has shown a substantial degree of direct reprogramming of cancer cells via the RICCS into selective cell lineages. Interestingly, evidences have suggested that functional activities of protein translation have no effect on transdifferentiation activity [17,28]. Therefore, in future, a wide range ribosome interaction analysis in RICCS can give a hint of mechanism of cancer cell reprogramming by ribosome incorporation.

Our qPCR analysis has given a glimpse of alteration in expression of cancer cell signature markers and cell cycle/proliferation markers. CXCR4 and EGFR is associated with many cancer progression including non-small cell lung carcinoma (NSCLC) [23,24,29]. Expression of EGFR and CXCR4 in A549 completely differed at corresponding D7 and D14 samples. Aberrant expression of cell cycle/proliferation markers such as Ki67 and CycD1 are often linked to tumor progression [30,31]. In A549

D7 sample, Ki 67 increased gradually but CycD1 showed a fall out at day 14. At day 14, while Ki67 was the highest suggesting an active cell cycle progression but CycD1 was the lowest suggesting an arrest in G0/G1 to S phase transition. This unusual gene expression pattern indicates the presence of an unknown mechanism where cells express ki67 but unable to proliferate instead fall into quiescent state by reducing CycD1. These observations have suggested that, ribosome incorporation in cancer cells alters the expression of cancer cell signature markers as well as cell cycle regulating markers in RICCSs at different time point. From tumor formation assay, we cannot conclude whether ribosome induces tumor growth progression or inhibition except a little change in tumor weight. The tremendously dynamic tumor microenvironment and the presence of heterogeneity are major challenges to test any therapeutic effect in the tumor growth at *in vivo* set up [32]. Therefore, we recommend in future to optimize the conditions of *in vivo* tumor growth assay to observe the effect of the ribosome incorporation more precisely. In conclusion, our study provides an out of the box approach to reprogram cancer cells into adipocyte and osteoblast via spheroid formation.

Declaration of competing interest

The authors 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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