

# Lifeceramics-Treated Water Suppresses the Migration and Invasion of Human Cancer Cells and Has Antioxidative Effects

Kazuko Kita<sup>1\*</sup>, Masaki Fukuyo<sup>1</sup>, Bahityar Rahmutulla<sup>1</sup>, Atsushi Kaneda<sup>1,2</sup>, Nobuo Suzuki<sup>3\*</sup>

<sup>1</sup>Department of Molecular Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan

<sup>2</sup>Health and Disease Omics Center, Chiba University, Chiba, Japan

<sup>3</sup>Non-Profit Organization, Chiba Researchers Network for Health Care Promotion, Chiba, Japan

Email: \*kita@faculty.chiba-u.jp, \*nobuo@faculty.chiba-u.jp

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## Abstract

An increase in the production of reactive oxygen species (ROS) leads to the promotion of ROS-dependent cancer cell proliferation, migration, invasion, and metastasis, and it has been reported that treatment of metastatic cancer cells with ROS scavengers suppresses their metastatic potential. Using cultured human cells, we have previously reported the antioxidative effects of lifeceramics (LC)-treated water (LC water) prepared by mixing MiliQ water with LC particles comprising zeolite and oyster shells. In this study, we investigated effects of culture medium prepared using LC water (LCM) on proliferation, migration, and invasion of human cancer cells, along with the accumulation of ROS. We found that migratory and invasive capacities, assessed *in vitro* using Transwell assays, were lower in LCM-cultured RKO colorectal and MKN45 gastric cancer cells than in the cancer cells cultured in control medium prepared with MilliQ water (ContM). Using a fluorescence probe, we detected reductions in both intracellular ROS accumulation and H<sub>2</sub>O<sub>2</sub>-induced ROS production in the LCM-cultured cells compared with the ContM-cultured cells. Furthermore, treatment of RKO cells with the antioxidant *N*-acetyl-L-cysteine resulted in a suppression of migration and invasion. In addition, gene expression analysis based on RNA-seq revealed that genes down-regulated in the LCM-cultured cancer cells compared with the ContM-cultured cells were enriched in gene ontology terms related to oxidative stress, suggesting a reduction in oxidative stress in the LCM-cultured cells. Some of these down-regulated genes are involved in oxidative stress-induced migration, invasion, or epithelial-mesenchymal transition in cancer cells. Collectively, our findings in this study indicate that exposure to LCM has migration-/invasion-suppressive and antioxidative effects in the two human cancer cells

examined. Furthermore, these findings suggest that the antioxidative effects of LC water contribute to the migration-/invasion-suppressive effects of LCM culture in association with the down-regulation of genes involved in oxidative stress-induced cancer progression.

### Keywords

Lifeceramics, Cancer Cells, Suppression of Migration and Invasion, Reactive Oxygen Species, Antioxidative Effects

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## 1. Introduction

Oxidative stress, which is typically induced in response to an excessive generation of reactive oxygen species (ROS), activates signaling pathways that contribute to cancer development by promoting proliferation, invasion, angiogenesis, and metastasis of cancer cells [1]-[3]. Under a limited availability of oxygen, some cancer cells depend on anaerobic glycolysis uncoupled from oxidative phosphorylation, a pathway associated with the production of ROS [4] [5]. However, in certain types of cancer cells, glycolysis occurs even in the presence of sufficient oxygen, which is a process referred to as aerobic glycolysis or the Warburg effect, and there is evidence to indicate that aerobic glycolysis can often be reverted to oxidative phosphorylation [4] [6]. For example, Porporato *et al.* have reported that this type of reversion involves excessive mitochondrial respiration and an increase in ROS production, leading to the promotion of ROS-dependent cancer cell migration, invasion, clonogenicity, and metastasis [4]. Moreover, these authors found that antioxidants and mitochondrial superoxide scavengers blocked the migration of cancer cells, and suggested that this suppression contributed to preventing the metastatic dissemination of cancers via the pharmacological scavenging of ROS. Other authors have reported that mitochondrial respiration is conducive to enhancing the migratory/invasive capacities of cancer cells [7], and that mutations in mitochondrial DNA associated with an excessive production of ROS may contribute to the high metastatic potential of cancer cells [8]. They also demonstrated that treatment of metastatic cancer cells with ROS scavengers suppressed their metastatic potential. Hirakawa *et al.*, found that ascorbic acid suppressed the ROS-potentiated invasive capacity of rat ascites hepatoma cells and suggested that the antioxidative activity of ascorbic acid is involved in the invasion-suppressive effect on the hepatoma cells [9].

Previous studies have assessed the antioxidant properties of alkaline electrolyzed water [10] [11]. We have previously investigated the effects of lifeceramics (LC)-treated water (LC water) on cellular responses to oxidative stress, using cultured human cells with high susceptibility to stresses. Lifeceramics is a material prepared from zeolite and powdered oyster shells under high temperature and pressure [12] [13]. The cells cultured in medium prepared with LC water (LCM)

showed an enhanced resistance to H<sub>2</sub>O<sub>2</sub>- and X ray-induced cell death compared with the cells cultured in medium prepared with MilliQ water (ContM) [14]. H<sub>2</sub>O<sub>2</sub> and X ray cause oxidative stress, and following H<sub>2</sub>O<sub>2</sub> treatment ROS levels actually increased in the cells cultured in ContM, whereas the levels in the cells cultured in LCM were lower than those in the control group [15]. The findings of these studies thus indicate that culturing cells in LCM can contribute to suppression of ROS accumulation and reduction of oxidative stress in the human cells examined, thereby providing evidence of the antioxidative effects of LC water *in vitro*. Moreover, using hyperlipidaemic rats, we have similarly demonstrated the antioxidative effects of LC water *in vivo* [16].

These previous findings pertain to the antioxidative effects of LC water in non-cancerous human cells. In this study, we investigated effects of LC water on proliferation, migration, and invasion, as well as ROS accumulation, in human cancer cells, and found that suppressive effects of LC water on migration and invasion and the accumulation of ROS in the cancer cells.

## 2. Materials and Methods

### 2.1. Cells and Culture Conditions

RKO and SW480 colorectal cancer (CRC) cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and MKN45 gastric cancer cell line was obtained from the Japanese Cancer Research Resources Cell Bank (JCRB, Tokyo, Japan). In addition, we used MIA PaCa-2 pancreatic cancer cell line [17]. RKO and MIA PaCa-2 cells were cultured in Eagle's minimum essential medium (Eagle's MEM; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) supplemented with penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 10% (v/v) foetal bovine serum (FBS; Regular; Collected in Brazil, Processed in UK, Corning, NY, USA). MKN45 and SW480 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI; FUJIFILM Wako Pure Chemical Corp.), containing penicillin-streptomycin and 10% (v/v) FBS. All cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere.

To prepare LC water, we mixed LC powder (Wedge Co., Ltd., Fuji, Japan) with MilliQ water at a final concentration of 0.03%. The mixture was allowed to stand overnight at 4°C, and then LC water was collected as supernatants following centrifugation of the mixture. The main elements present in the LC powder were calcium and magnesium. LC water, prepared from MilliQ water mixed with the low concentration of LC powder, as described above, contained mainly the three minerals (sodium 1.8 mg/L, magnesium 1.9 mg/L, and calcium 15 mg/L) and was weakly alkaline (around pH 10) [18]. ContM and LCM were prepared by dissolving medium powder (Eagle's MEM or RPMI 1640 medium; Shimadzu Diagnostics Corp., Tokyo, Japan) in MilliQ water and LC water, respectively, according to the manufacturer's protocol. The pH of the medium was adjusted by adding an 8% NaHCO<sub>3</sub> solution of the appropriate volume to each medium and further adding a small amount of 1 N HCl solution to LCM for the RPMI 1640 medium. We

confirmed that the pH of each medium was maintained around 7.4 in a 5% CO<sub>2</sub> atmosphere during cell culture, and we examined the effects of culturing cancer cells in these media.

## 2.2. WST-8 Cell Proliferation Assay

Cells pre-cultured in ContM or LCM for 5 days were harvested during the exponential phase of growth, seeded in 96-well plates ( $1.2 \times 10^3$  cells/well) with the respective media, and thereafter cultured for 4 days. Assessments of the numbers of viable cells were performed daily based on the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] method using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's protocol. Briefly, WST-8 solution was added to the cultured cells in each well, and following incubation for 3 h, absorbance of the well contents was measured at 450 nm using a microplate reader (Synergy LX multi-mode plate reader; BioTek Instruments Inc., Winooski, VT, USA).

## 2.3. *In Vitro* Migration and Invasion Assays

The migratory and invasive capacities of cancer cells were evaluated using Corning® BioCoat™ Transwell chambers (#354578 and #354480, respectively; Corning) with 24-well inserts and 8-mm pore-sized membranes, following the manufacturer's protocol. Briefly, following pre-culture in ContM or LCM for 6 days, RKO and MKN45 cells were suspended in serum-free each medium and plated (RKO cells:  $2.5 \times 10^5$  cells/well; MKN45 cells:  $5 \times 10^5$  cells/well) on uncoated upper chambers for migration assay and on Matrigel-coated upper chambers for invasion assay. RKO cells were also cultured in ContM with or without 1 mM *N*-acetyl-L-cysteine (NAC) (FUJIFILM Wako Pure Chemical Corp.) for 3 days, and plated on both chambers, as described above. SW480 cells ( $1.3 \times 10^5$  cells/well in both assays) and MIA PaCa-2 cells ( $0.5 \times 10^5$  cells/well in the migration assay and  $2.5 \times 10^5$  cells/well in the invasion assay) were similarly plated following pre-culture. Each medium containing 20% serum was used as a chemoattractant in the lower chambers. Following incubation for 48 h, non-migrating and non-invading cells were gently removed from the upper surface of the membrane using a cotton-tipped swab. Migrating and invading cells through the membrane were fixed and stained with Diff-Quik staining solution (#16920; Sysmex, Hyogo, Japan) and the numbers of cells in each well were counted in approximately 10 randomly selected fields of view under a microscope using BZ-X analysis software (Keyence, Osaka, Japan). The mean of the numbers of migratory/invasive cells per field of view was taken to be indicative of the migratory and invasive capacities, respectively, and the capacities of the LCM-cultured cells were expressed as a ratio relative to those of the ContM-cultured cells.

## 2.4. Reactive Oxygen Species Measurement

Intracellular ROS levels were assessed using CellROX Green Reagent (C10444,

Invitrogen, Waltham, MA, USA) probe, according to the manufacturer's instruction. Briefly, following pre-culture in ContM or LCM for 6 days, the cells were suspended in the respective media, seeded in 96-well plates ( $1 \times 10^4$  cells/well), and cultured for 24 h.  $H_2O_2$  solution (FUJIFILM Wako Pure Chemical Corp.) was added at the indicated doses followed by incubation for 15 min, after which, CellROX green reagent was added at 6  $\mu M$ , with further incubation for 25 min. Following incubation, the cells were washed with PBS, and fluorescence (Ex/Em = 485/528 nm) was analysed using a fluorescence microplate reader (Synergy HTX multi-mode plate reader; BioTek Instruments, Inc.). The cells in each well were also stained with a crystal violet dye, and absorbance was measured at 595 nm using the Synergy LX microplate reader. The intensity of ROS fluorescence was normalized with respect to the absorbance at 595 nm, and the normalized ROS values were considered indicative of ROS levels. The levels obtained for each treatment were expressed relative to those of the ContM culture condition without  $H_2O_2$  treatment, as the relative ROS intensity. No decrease in cell viability was observed, even under the  $H_2O_2$  existence, at least during the measurement period.

## 2.5. Gene Expression Analysis

RNA was extracted from cancer cells cultured in ContM or LCM for 6 days using a Direct-zol<sup>TM</sup> RNA Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol, and gene expression was examined based on RNA-seq analysis. Libraries for RNA-seq samples were prepared using a TruSeq Stranded mRNA Sample Prep Kit (Illumina Inc., San Diego, CA, USA) following the manufacturer's protocol. Deep sequencing was performed using the Illumina NovaSeq 6000 platform. More than 5 million reads per sample were acquired through the RNA-seq analysis. STAR (version 2.6.0c) [19] was used to map FASTQ reads to the hg38 reference genome and RSEM (v1.3.1) [20] was used for transcript abundance quantification. Gene expression levels are expressed as transcripts per million mapped sequence reads (TPM). With respect to TPM fold-change determination, TPM with values  $<1.0$  were all designated '1.0', given that these values are considered to be less reliable. Genes down- and up-regulated in response to culturing in LCM were selected based on  $\log_2$  TPM fold-change (LCM versus ContM)  $<-0.25$  and  $>0.25$ , respectively, in both RKO and MKN cells, and the four cell lines examined were sorted into two groups, LCM-responsive cells and LCM-non-responsive cells, by two-way hierarchical clustering of the genes selected using the cutoff range.

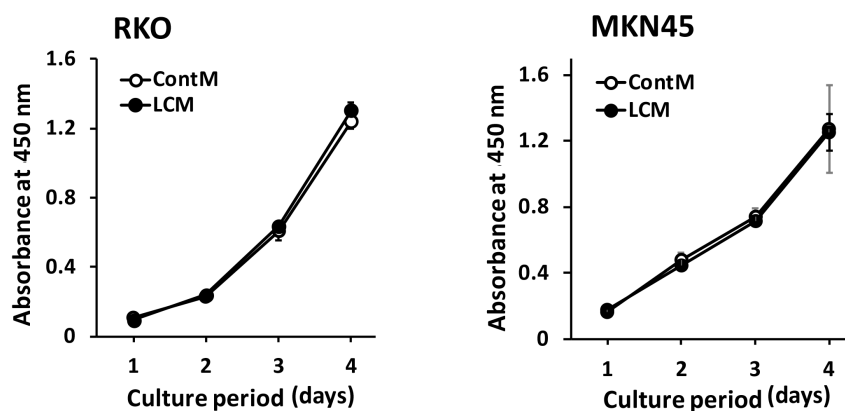
## 2.6. Maintaining the Integrity of the Specifications

Data are presented as mean  $\pm$  standard deviation (SD). Differences among groups were assessed using Student's *t*-test, with statistical significance set at  $P < 0.05$ . Heat maps were drawn using Java TreeView software (<http://jtreeview.sourceforge.net/>), and gene ontology (GO) enrichment analysis was performed using the gene annotation tool in the Metascape web application [21] (<http://metascape.org>).

### 3. Results

#### 3.1. Effects of LCM Culture on the Rates in Cancer Cell Proliferation

To examine effects of LCM culture on the proliferation of cancer cells, we pre-cultured RKO and MKN45 cells in ContM or LCM for 5 days, and thereafter assessed cell growth under each culture condition using WST-8 assay over a 4-day time course. During the experimental period, we detected no significant differences between the assessed culture conditions with respect to the rates of cancer cell proliferation (Figure 1), thereby indicating that LCM has no appreciable effects on cell proliferation. Similar results were obtained for SW480 and MIA PaCa-2 cells (Figure S1). In addition, for the pre-culture period, growth rates determined by cell number counting were not significantly altered by LCM culture in all four assessed cancer cell lines (data not shown).



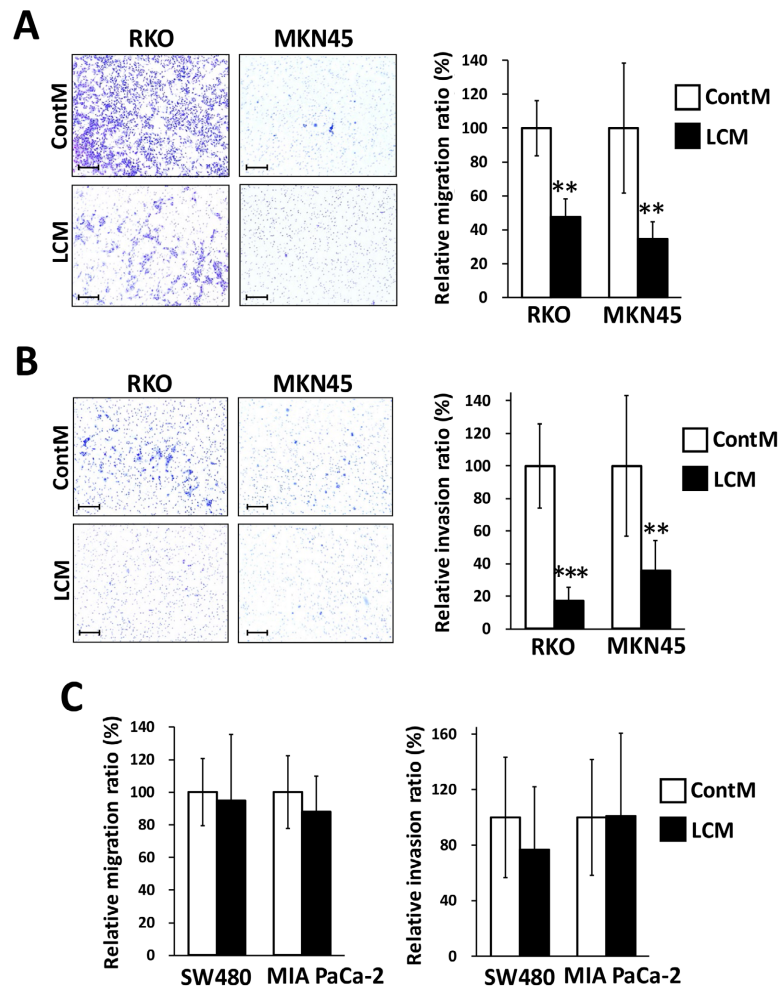
**Figure 1.** The effects of LCM culture on cancer cell proliferation.

Following pre-culture in ContM or LCM for 5 days, the rates of RKO and MKN45 cell proliferation were estimated using WST-8 assay over a 4-day time course. Data are presented as mean  $\pm$  standard deviation (SD) of three wells of the 96-well plate,  $n = 3$ .

#### 3.2. Effects of LCM Culture on the Migration and Invasion in Cancer Cells

We examined the migratory and invasive capacities of the four cancer cell lines, using Transwell migration and invasion assays, which revealed that compared with MKN45 cells, RKO cells were characterized by higher capacities of both invasion and migration (Figure 2(A), Figure 2(B)); for RKO and MKN45 cells, when cultured in ContM, we obtained mean values (from four wells of the Transwell chamber) of  $1530 \pm 249$  and  $25.8 \pm 9.83$  for the numbers of migratory cells and  $88.3 \pm 22.8$  and  $32.4 \pm 14.0$  for the numbers of invasive cells per field of view, respectively. Notably, for both cell lines, the migratory and invasive capacities decreased significantly in the LCM-cultured cells compared to the ContM-cultured cells (Figure 2(A), Figure 2(B)); the counts of migratory cells decrease

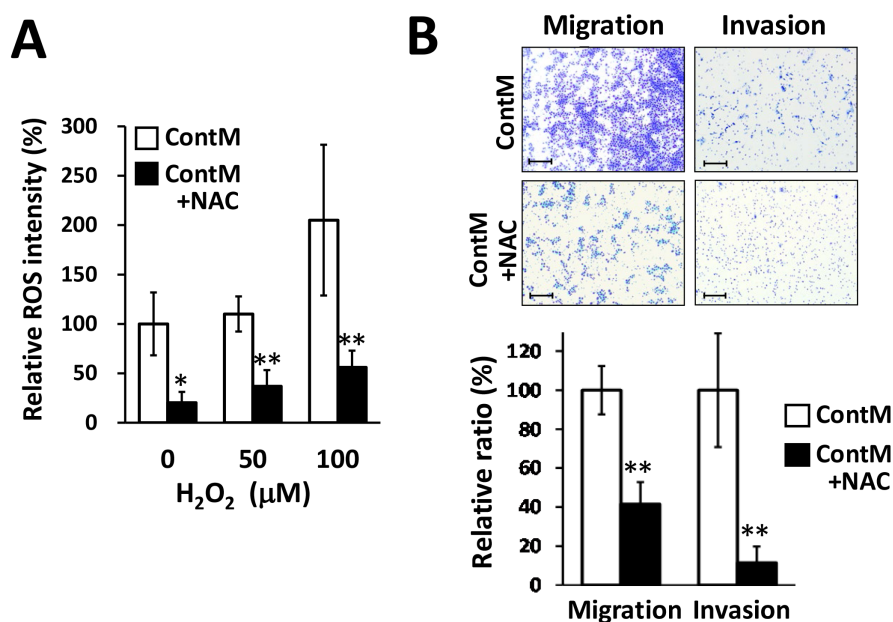
to approximately 50% and 35%, and those of invasive cells decreased to approximately 20% and 35%, in RKO and MKN45 cells, respectively, thereby providing evidence for the suppression of cancer cell migration and invasion by LCM in both cell lines. Contrastingly, in the case of SW480 and MIA PaCa-2 cells, there was no significant differences between the ContM and LCM culture conditions in the either migratory or invasive capacity (Figure 2(C)).



**Figure 2.** The effects of LCM culture on cancer cell migration and invasion. RKO and MKN45 cells were pre-cultured in ContM or LCM for 6 days, after which, the migratory (A) and invasive (B) capacities were evaluated as described in the Materials and Methods section. Left; representative images of the lower surface of the Transwell membranes are shown ( $\times 10$  magnification; scale bars, 200  $\mu\text{m}$ ). Right; the numbers of migrating (A) and invading (B) cells under both culture conditions were counted in selected fields of view under the microscope and statistically analysed. The migratory and invasive capacities of LCM-cultured cells are expressed as the ratio relative to those of ContM-cultured cells. In both assays, four wells of the Transwell chamber were used for the respective cells. Data are presented as mean  $\pm$  standard deviation (SD),  $n = 4$ . \*\* $P < 0.01$ , \*\*\* $P < 0.001$  for LCM culture vs. ContM culture. SW480 and MIA PaCa-2 cells were similarly pre-cultured in ContM and LCM, and the migratory (C, left) and invasive (C, right) capacities were evaluated as described above. In both assays, two wells of the Transwell chamber were used. Data are presented as mean  $\pm$  standard deviation (SD),  $n = 2$ .

### 3.3. Effects of LCM Culture on ROS Accumulation in Cancer Cells

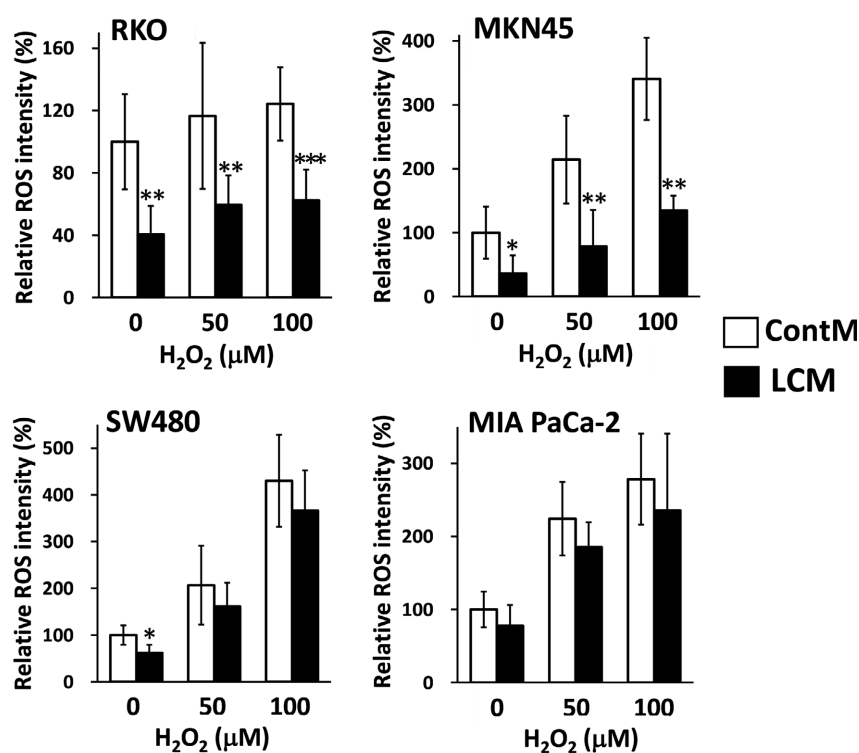
With respect to oxidative stress, we initially examined whether NAC, a typical antioxidant, could inhibit the migration and/or invasion of RKO cells, which we established to have higher migratory/invasive capacities compared with MKN45 cells. NAC inhibited both of the intracellular accumulation of ROS associated with cell metabolism in the absence of H<sub>2</sub>O<sub>2</sub> treatment and H<sub>2</sub>O<sub>2</sub> (50 μM and 100 μM)-induced ROS production (Figure 3(A)), and also suppressed migration and invasion in the cells (Figure 3(B)). These findings show that under the assessed assay conditions, antioxidants could suppress the migration and invasion of the cancer cells.



**Figure 3.** The effects of NAC on ROS levels and the migratory and invasive capacities of RKO cells. RKO cells were pre-cultured for 3 days in ContM or the medium containing 1 mM NAC (ContM+NAC), after which, ROS levels were measured as described in the Materials and Methods section, and the levels of ROS are expressed relative to those in the cells cultured in ContM without H<sub>2</sub>O<sub>2</sub> treatment, as the relative ROS intensity (A). Three wells of the 96-well plate were used for each concentration of H<sub>2</sub>O<sub>2</sub>. Data are presented as mean ± standard deviation (SD),  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$  for ContM+NAC vs. ContM. The migratory and invasive capacities were analysed for each culture condition (B). Upper; representative images of the lower surface of the Transwell membranes are shown ( $\times 10$  magnification; scale bars, 200 μm). Lower; the migratory and invasive capacities of the cells cultured in ContM+NAC are expressed as the relative ratio to those of ContM-cultured cells. In both assays, three wells of the Transwell chamber were used. Data are presented as mean ± standard deviation (SD),  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$  for ContM+NAC vs. ContM.

Subsequently, we compared the accumulation of ROS in the LCM- and ContM-cultured cells. In the absence of H<sub>2</sub>O<sub>2</sub> treatment, the levels of ROS in RKO and MKN45 cells cultured in LCM were found to be less than 50% of those detected in ContM-cultured cells (Figure 4 (upper)), thus tending to indicate an inhibition

of ROS production in both cell lines following exposure to LCM. Similarly, when treated with H<sub>2</sub>O<sub>2</sub> at 50 μM and 100 μM, the ROS levels increased in both cell lines when cultured in ContM, but the increased levels were also significantly suppressed in the LCM-cultured cells (Figure 4 (upper)). These findings thus indicate that culturing in LCM can inhibit not only the intracellular accumulation of ROS but also ROS produced in response to the oxidative stress by the H<sub>2</sub>O<sub>2</sub> treatment in both cancer cells. On the other hand, in SW480 cells the intracellular accumulation of ROS was only weakly inhibited by LCM culture, whereas culturing under these conditions had no significant effects regarding H<sub>2</sub>O<sub>2</sub>-induced ROS production (Figure 4 (lower)). Moreover, in the case of MIA PaCa-2 cells, culturing in LCM inhibited neither intracellular ROS accumulation nor H<sub>2</sub>O<sub>2</sub>-induced ROS production (Figure 4 (lower)).

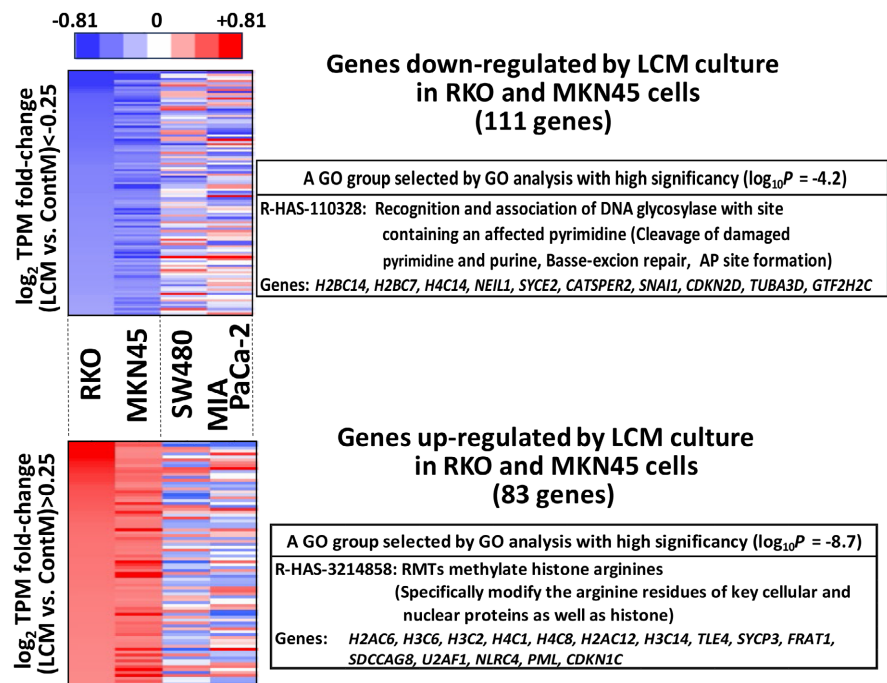


**Figure 4.** The effects of LCM culture on the accumulation of ROS. Following pre-culture in ContM or LCM for 6 days, levels of intracellular ROS in the four assessed cancer cell lines, with and without H<sub>2</sub>O<sub>2</sub> treatment, were determined based on measurements of fluorescence intensity using CellROX Green Reagent. The levels of ROS are expressed in terms of fluorescence intensity relative to those in the ContM culture condition without H<sub>2</sub>O<sub>2</sub> treatment, as the relative ROS intensity. Data are presented as mean ± SD of three wells of the 96-well plate,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  for LCM culture vs. ContM culture.

### 3.4. Changes in Gene Expression in Response to LCM Culture in Cancer Cells

To determine the effects of LC water on gene expression, we analysed changes in gene expression in the four assessed cancer cell lines (RKO, MKN45, SW480, and

MIA PaCa-2), cultured in LCM or ContM based on RNA-seq. Among 17,129 genes expressed in the four cell lines, 111 and 83 genes were selected as down- and up-regulated, respectively, in response to culturing in LCM culture in both of RKO and MKN45 cells (Figure 5). Heatmaps generated to visualize patterns in gene expression change revealed certain differences between the two cell line groups (Figure 5 (left panel)); the group consisting of RKO and MKN45 cells, being responsive to the migration-/invasion-suppressive and antioxidative effects of culturing in LCM, and the other group consisting of SW480 and MIA PaCa-2 cells, being either weakly responsive or non-responsive to the effects.



**Figure 5.** The effects of LCM culture on gene expression in cancer cells. RNA was extracted from the four assessed cancer cell lines cultured in ContM or LCM for 6 days, and gene expression was analysed based on RNA-seq, as described in the Materials and Methods section. Genes down- and up-regulated in response to culturing in LCM were selected on the basis of a  $\log_2$  TPM fold-change (LCM versus ContM)  $< -0.25$  and  $> 0.25$ , respectively, in both of RKO and MKN45 cells. In heatmaps, changes in the levels of 111 down-regulated and 83 up-regulated genes in the four assessed cell lines are presented in order of the rate of change in RKO cells in left upper and lower panels, respectively. GO groups selected based on GO analysis from the down- and up-regulated genes with highest significance are shown in right upper and lower panels, respectively, with genes classified in the respective GO groups. No other GO groups with high significance were selected from either down-regulated genes or up-regulated genes.

The 111 down-regulated genes were most significantly enriched in the GO term ‘Recognition and association of DNA glycosylase with site containing an affected pyrimidine’ ( $P = 1 \times 10^{-4.2}$ ) (Figure 5 (right upper panel)). The functions of the GO group (Dn-GO) include base-excision repair involved in the repair of oxidative DNA damage and oxidative stress-induced cell cycle arrest, and *NEIL1* and

*CDKN2D* in Dn-GO are the typical genes playing roles in the response to oxidative stress (Table 1). These findings indicate that in the two LCM-responsive cancer cells, there was a reduction in the response to oxidative stress in the culture condition of LCM compared with that of ContM, which is taken to be indicative of antioxidative effects by LCM culture in the two cancer cells. Furthermore, some of genes classified in the Dn-GO group, namely, *SNAIL*, *TUBA3D*, *GTF2H2C*, and *SYCE2*, have been reported to be up-regulated in certain cancer cells and function in cancer progression, including the promotion of cancer cell proliferation, metastasis, migration/invasion, or epithelial-mesenchymal transition (EMT) (Table 1). In addition, we identified the top 10 down-regulated genes in RKO (Dn-RK) and MKN45 (Dn-MK) cells, respectively, some of which are also associated with the progression of cancer (Table 1).

The 83 up-regulated genes were most significantly enriched in the GO term 'RMTs methylate histone arginines' ( $P = 1 \times 10^{-8.7}$ ) (Figure 5 (right lower panel)), thereby tending to indicate that LCM-induced gene expression involves epigenetic modification. Some of genes in the GO group (Up-GO), including *TLE4*, *PML*, *CDKN1C*, and *U2AF1*, have been reported to be down-regulated in certain cancer cell lines, including CRC cells, and have been established to play roles in cancer suppression, such as the inhibition of cancer cell proliferation, metastasis, migration/invasion, or EMT (Table 2). Other genes among the top 10 up-regulated genes in RKO (Up-RK) and MKN45 (Up-MK) cells have also been established to function in cancer suppression (Table 2).

#### 4. Discussion

In this study, we found that culturing in LCM prepared with LC water had migration-/invasion-suppressive effects on RKO and MKN45 cancer cells (Figure 2). Similar migration-suppressive effects were also observed in a wound-healing assay conducted using these two cell lines (data not shown). In addition, we found the antioxidative effects of LCM in both cell lines, including inhibition of the intracellular accumulation of ROS and H<sub>2</sub>O<sub>2</sub>-induced ROS production (Figure 4). In contrast, we detected neither migration-/invasion-inhibitory effects nor notable antioxidative effects of LCM in SW480 and MIA PaCa-2 cells (Figure 2 and Figure 4). The antioxidative effects of LCM culture in RKO and MKN45 cells were also suggested from the result of gene expression analysis (Figure 5). Furthermore, we demonstrated the migration-/invasion-suppressive effects of the antioxidant reagent NAC in RKO cells (Figure 3). Collectively, these findings suggest that the antioxidative effects of LC water contribute to the migration-/invasion-suppressive effects of LCM culture on RKO and MKN45 cells. The detailed differences in the physicochemical properties of ContM and LCM are unclear. However, the property concerned with viability and growth of the cells seemed to make little difference, because for the culture period in this study, growth rates were not significantly altered by LCM culture in any of the four cancer cell lines (Figure 1 and Figure S1).

**Table 1.** The functions of genes selected as down-regulated genes in LCM culture.

Functions	Genes	Group	Refs.
Oxidative stress response	Base excision repair	<i>NEIL1, H2BC14, H2BC7, H4C14</i>	Dn-GO
	Oxidative stress-induced cell cycle arrest	<i>CDKN2D, H2BC14, H2BC7, H4C14</i>	Dn-RK [27] [28] Dn-MK
	Promotion of cancer cell proliferation, metastasis, migration, invasion or EMT	<i>SNAI1, TUBA3D, GTF2H2C, SYCE2</i> <i>BOLA2, PHOSPHO2-KLHL23, PILRB</i> <i>EIF3C</i>	Dn-GO [29]-[36] Dn-RK [37]-[39] Dn-MK [40] [41]

EMT: epithelial-mesenchymal transition. Dn-GO: The group of down-regulated genes in RKO and MKN45 cells selected based on GO analysis is shown in **Figure 5**. *H2BC7* and *H4C14* in Dn-GO are also classed in the Dn-RK and Dn-MK groups, respectively. Dn-RK: The top 10 down-regulated genes in RKO cells ( $\log_2$  TPM fold change (LCM/ContM) = -6.6 - -0.71). Dn-MK: The top 10 down-regulated genes in MKN45 cells ( $\log_2$  TPM fold change (LCM/ContM) = -1.1 - -0.53).

**Table 2.** The functions of genes selected genes up-regulated in LCM culture.

Functions	Genes	Group	Refs.
Cancer suppression	Suppression of cancer cell proliferation, metastasis, migration, invasion or EMT	<i>TLE4, PML, CDKN1C, U2AF1</i>	Up-GO [42]-[46] Up-RK
	Tumor suppressor	<i>HSD17B6</i>	Up-RK [47]
		<i>SPTY2D1OS</i>	Up-MK [48]

Up-GO: The group of up-regulated genes in RKO and MKN45 cells selected based on GO analysis is shown in **Figure 5**. *U2AF1* in Up-GO is also classed in the Up-RK group. Up-RK: The top 10 up-regulated genes in RKO cells ( $\log_2$  TPM fold change (LCM/ContM) = 0.71 - 2.1). Up-MK: The top 10 upregulated genes in MKN45 cells ( $\log_2$  TPM fold-change (LCM/ContM) = 0.74 - 2.0).

At present, it is still unclear as to why neither any notable antioxidative effects nor migration-/invasion-suppressive effects were detected in LCM-cultured SW480 and MIA PaCa-2 cells. However, when we compared arbitrary ROS intensities, rather than relative values, between ContM-cultured RKO and MIA PaCa-2 cells, which were both cultured in the same medium (Eagle's MEM), the intracellular levels of ROS in the absence of H<sub>2</sub>O<sub>2</sub> treatment appeared to be slightly higher in MIA PaCa-2 cells than in RKO cells (data not shown). Following H<sub>2</sub>O<sub>2</sub> treatment, a more pronounced increase in the relative levels of ROS was observed in MIA PaCa-2 cells than in RKO cells (**Figure 4**). MKN45 and SW480 cells, which were both cultured in the same medium (RPMI 1640 medium), were found to have comparable intracellular levels of ROS (data not shown); however, we observed a slightly higher apparent increase in ROS levels in response to treatment with H<sub>2</sub>O<sub>2</sub> in SW480 cells than in MKN45 cells (**Figure 4**). It is thus conceivable that the extents of oxidative stress are more pronounced in MIA PaCa-2 and SW480 cells than in RKO and MKN45 cells, and the antioxidative activity attributable to LCM culture may be insufficient to suppress the oxidative stress in SW480 and MIA PaCa-2 cells.

The antioxidative activity of electrolyzed reduced water has been established to be attributable to the scavenging of ROS [22]-[24]. Interestingly, two different types of electrolyzed reduced water have been reported, one of which scavenges ROS directly *in vitro*, whereas the other type is associated with indirect scavenging of ROS mediated via interactions with other antioxidative compounds both intra- and extracellularly [22] [23]. In this regard, we have previously reported that *in vitro*, LC water does not scavenge ROS directly but instead contributes to enhancing the antioxidative activity of low dose ascorbic acid, thus suggesting that the action of LC water is of the latter type described above [15], although the precise mechanisms underlying its antioxidative effects have yet to be elucidated. The type of antioxidative activity of LC water may be also associated with difference in the effects of LCM culture between the two groups of cancer cells. Alternatively, the observed difference in response to the migration-/invasion-suppressive effects of LCM could be associated with a difference in the *TP53* status between the two cell groups, as whereas both RKO and MKN45 cells express wild-type *TP53*, SW480 and MIA PaCa-2 cells have mutated *TP53* [17]. That's because p53 has been reported to have the antioxidative function via the up-regulation of genes encoding antioxidative proteins [25], along with roles in regulating cellular processes, including the inhibition of invasion and metastasis [26].

It has also been reported that mitochondrial respiration is conducive to the migratory/invasive behaviour of cancer cells, and that excessive mitochondrial respiration, and hence heightened levels of ROS production, contribute to the promotion of ROS-dependent cancer cell migration, invasion, clonogenicity, and metastasis [4] [6]-[8]. Pre-treatment of highly metastatic cancer cells with ROS scavengers has been shown to suppress their metastatic potential [9], and pharmacological scavenging of ROS has been suggested to prevent the expansion of metastatic cancers [4]. In the present study, we found that SW480 and MIA PaCa-2 cells appeared to be characterized by more pronounced migratory or invasive activity than RKO and MKN45 cells, particularly MIA PaCa-2 cells, for which these activities were approximately 2.5- and 4.3-fold higher, respectively, than those in RKO cells. However, the migration-/invasion-suppressive activities of LCM appeared to be not effective against SW480 and MIA PaCa-2 cells with these higher progression activities. On the basis of our findings in this study, and those reported previously [4] [9], we speculate that the migration-/invasion-suppressive activity of LC water might be more effective in preventing the transition of cancer cells to highly metastatic types than suppressing the metastatic potential of highly metastatic cancers, even though the activity of LC water was observed only *in vitro* in the two responsive cell lines and seemed cell line-specific so far.

From the gene expression analysis, the 111 down-regulated genes were selected in the two LCM-responsive cancer cell lines, RKO and MKN45, and these genes were enriched in the Dn-GO group associated with the response to oxidative stress (Figure 5 and Table 1), which tends to be consistent with the antioxidative effects of LCM culture on these two cell lines. Among the genes classified in Dn-GO,

*NEIL1* and *CDKN2D* encode proteins typically involved in the response to oxidative stress, including the repair of oxidative DNA damage and oxidative stress-induced cell cycle arrest, respectively (**Table 1**) [27] [28]. In addition, other genes or gene products in this group have been reported to be involved in oxidative stress and the progression of cancer (**Table 1**). For example, *SNAI1*, which contributes to carcinogenesis via EMT, has been reported to facilitate the progression of CRC [29] and promote the migration and invasion of thymic epithelial tumors cell lines [30]. Similarly, Wen *et al.* found that the long non-coding RNA-XIST promoted the oxidative stress-induced migration, invasion, and EMT of osteosarcoma cells via up-regulation of *SNAI1* [31]. Furthermore, *TUBA3D* is the aggrephagy-related gene and encodes the different isoform of  $\alpha$ -tubulin that plays pivotal roles in intracellular architecture, cell migration, and signal transduction [32]. Aggrephagy, the process whereby protein aggregates are degraded, is among the responses to oxidative stress [33]. GTF2H2C, a component of the transcription factor TFIID complex, has been reported to be involved in the transcription-coupled repair of damaged DNA attributable to UV irradiation and oxidative stress [34], and has recently been identified as a potential marker for predicting the metastasis of non-small cell lung cancer [35]. Moreover, SYCE2, which contributes to the regulation of DNA damage repair, has been reported to be associated with cellular resistance to radiation or DNA-damaging treatments [36]. Collectively, these findings suggest that in the two LCM-responsive cancer cell lines assessed in this study, the migration-/invasion-suppressive effects of LCM may be mediated via down-regulation of these oxidative stress-related genes. In addition, genes classified in the Dn-RK or Dn-MK groups of down-regulated genes, including *BOLA2*, *PHOSPHO2-KLHL23*, *PILRB*, and *EIF3C*, have been reported to be involved in the metastatic phenotype, migration, and invasion of cancer cells (**Table 1**) [37]-[40]. *EIF3C* has also recently been identified as an oxidative stress-related gene [41].

The 83 up-regulated genes were enriched in the Up-GO group (**Figure 5** and **Table 2**), indicating that epigenetic modification-related pathways were potentially involved in changes in the gene expression in LCM-cultured RKO and MKN45 cells. For example, it has been found that lysine-specific demethylase 1 (LSD1) suppresses the expression of LSD1-target genes, including *TLE4* in Up-GO, via demethylation of histone H3 lysine4 at LSD1-target gene promoter region, and thereby enhances the invasion and metastasis of CRC [42]. However, the mechanisms whereby these epigenetic changes were induced by LCM culture are yet unclear. There are some other genes identified as being involved in the suppression of cancer cell proliferation and migration/invasion in the GO group and other two groups, Up-RK and Up-MK (**Table 2**). Among the products of those genes, PML and *CDKN1C* have been suggested to be associated with oxidative stress in cancer cells. PML has been known to function as a tumor suppressor in different types of cancers and to associate with suppression of metastatic progression in CRC [43]. Han *et al.* have reported that low-dose H<sub>2</sub>O<sub>2</sub> reduces the

number of PML bodies, thereby leading to mild oxidative stress-induced cell proliferation, and have suggested that this protein may contribute to the suppression of oxidative stress-induced cell proliferation and is plausibly associated with human cancers [44]. CDKN1C (p57(Kip2)) has been also identified as a candidate tumor suppressor, and Wang *et al.* have reported that the antioxidant compound curcumin contributes to the suppression of cell growth, migration, and invasion in glioma cells via up-regulation of *CDKN1C* [45]. Other genes classified in the Up-GO, Up-RK or Up-MK groups of up-regulated genes, including *U2AF1*, *HSD17B6*, and *SPTY2DIOS*, have been reported to be involved in the suppression of cancer progression (Table 2) [46]-[48].

Thus, on the basis of the foregoing findings, it can be speculated that the migration-/invasion-inhibitory effects of LCM culture on RKO and MKN45 cancer cells are associated with the down-regulation of the cancer-progressive genes and the up-regulation of the cancer-suppressive genes.

## 5. Conclusion

Collectively, our findings in this study suggest that the antioxidative activity of LC water contributes to the suppression of the migratory and invasive capacities of the human cancer cell types examined, although the precise mechanisms underlying this efficacy remain to be elucidated. These findings underscoring the potentially beneficial effects of LC water on human cancer cells *in vitro* may provide a foundation for the development of countermeasures to prevent the progression of cancer.

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## Authors' Contributions

During the research and preparation of the manuscript, Kazuko Kita (KK) and Nobuo Suzuki (NS) designed the study, as corresponding authors, and KK performed the experiments and analysed the experimental data. Bahityar Rahmutulla performed RNA-seq analysis, and Masaki Fukuyo and Atsushi Kaneda (AK) analysed the gene expression data. KK prepared the manuscript, and NS and AK revised the manuscript. All authors have read and agreed to publish this manuscript.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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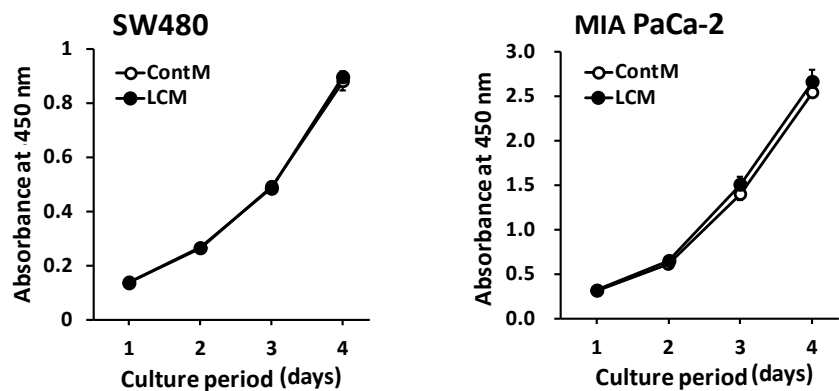
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## Supplementary Material



**Figure S1.** The effects of LCM culture on cancer cell proliferation. Following preculture in ContM or LCM for 5 days, the rates of SW480 and MIA PaCa-2 cell proliferation were estimated using WST-8 assay over a 4-day time course. Data are presented as mean  $\pm$  standard deviation (SD) of three wells,  $n = 3$ .