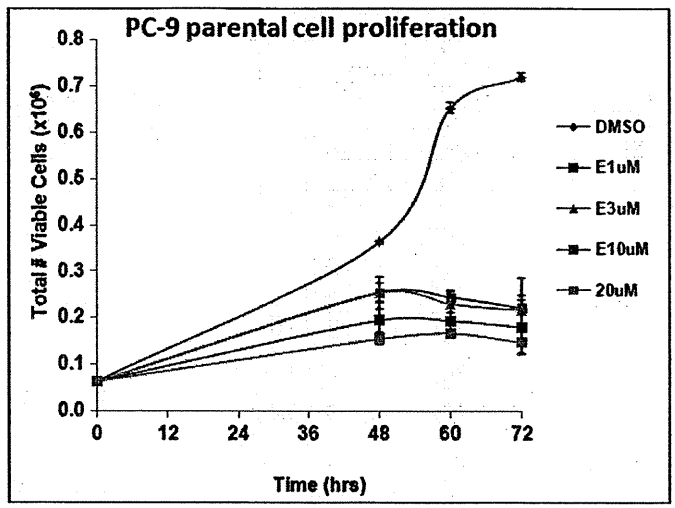
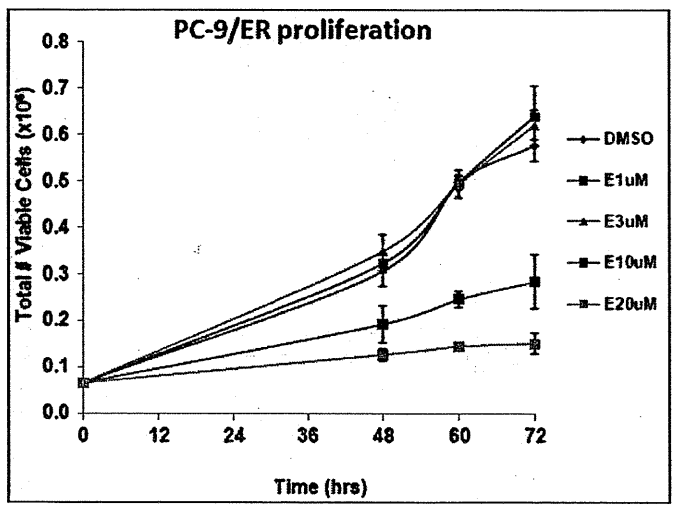
**Figure S5**


PC-9 parental cell proliferation
Total # Viable Cells (x10⁶)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
12
24
36
48
60
72
Time (hrs)
DMSO
E1uM
E3uM
E10uM
20uM


PC-9/ER proliferation
Total # Viable Cells (x10⁶)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
12
24
36
48
60
72
Time (hrs)
DMSO
E1uM
E3uM
E10uM
E20uM

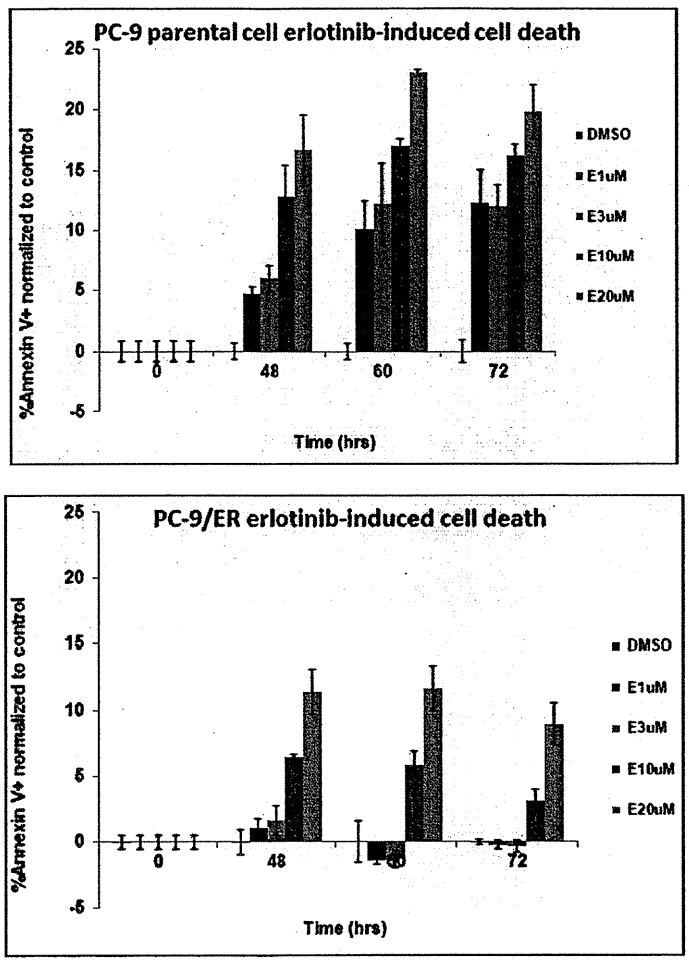



**Figure S5. Derivation of mathematical parameters.** Growth and death rates of PC-9 parental and PC-9/ER cells were determined by cell counting and annexin V/PI staining, respectively. Measurements were taken every 12 hours beginning 48 hours after the addition of drug. Data are plotted as the average of three replicates +/- standard deviation. Par- parental; ER- erlotinib resistant; E- erlotinib.

**Figure S6**

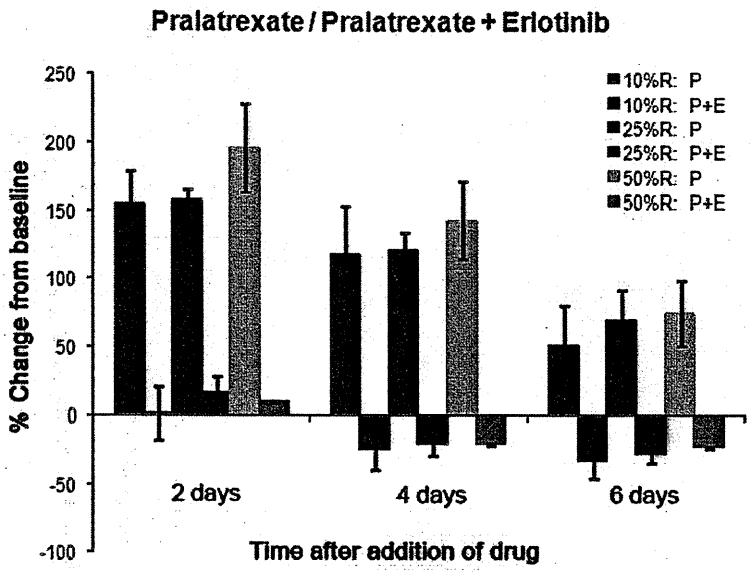



**Figure S6. Continuation of TKI therapy in cell populations with T790M-harboring clones leads to better tumor cell control.** PC-9/BR c1 resistant cells were diluted in parental cells at various concentrations (see **Figure 3A**) and randomized to receive chemotherapy (pralatrexate, 100 μM) or chemotherapy plus erlotinib (3 μM). In all cases, the TKI-chemotherapy combination was more efficacious at inhibiting cell growth.

**Figure S7**

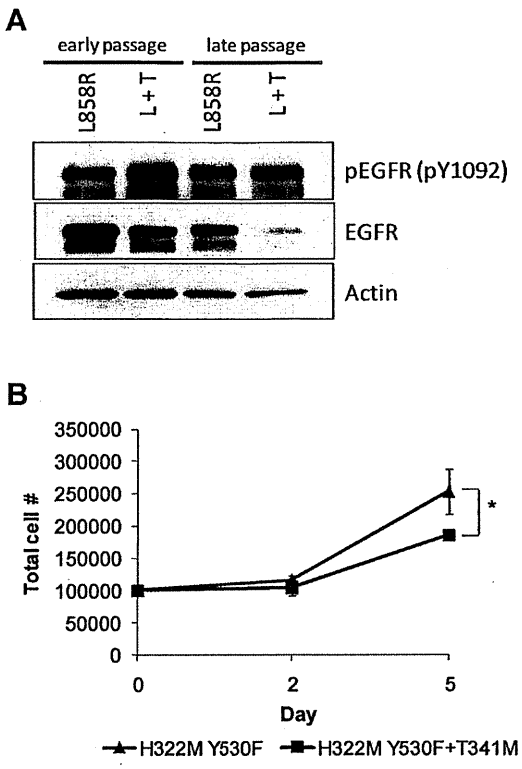



**Figure S7. Toxicity of "gatekeeper" mutations in other cell models. (A)** NR-6 cells transduced with either L858R or L858R in combination with T790M (L+T) spontaneously selected for lower levels of the double mutant EGFR over time, suggesting toxicity of the double mutant allele in these cells. **(B)** H322M cells were transfected with constitutively active SRC (Y530F) alone or in combination with the SRC gatekeeper mutation (Y530F+T341M). Cells with the double mutant SRC grew slower than the parental and single mutant cells. (* $p < 0.01$)

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# Randomized Phase II Study of Two Schedules of Carboplatin and Gemcitabine for Stage IIIB and IV Advanced Non-Small Cell Lung Cancer (JACCRO LC-01 Study)

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**Key Words**
Lung cancer · Randomized phase II study · Gemcitabine · Carboplatin · Thrombocytopenia

**Abstract**
***Background:*** Gemcitabine combined with carboplatin (CG) is one of the regimens used widely for advanced non-small cell lung cancer. Improvement in its toxicity may result in good clinical outcomes. ***Methods:*** A new schedule of gemcitabine and carboplatin (CG8) was compared with the standard one (CG1). Both are 3-weekly regimens, but carboplatin is administered on day 1 in CG1 and on day 8 in CG8. ***Results:*** The response rate of CG1 was 29.2%, which was higher than that of CG8 (22.2%). Median survival times in CG1 and CG8 were 348 and 455.5 days, respectively. Grade ≥3 leukopenia, thrombocytopenia and anemia were observed in 56.0, 72.0 and 36.0% of patients with CG1 and in 33.3, 25.9 and 14.8% of patients with CG8, respectively. Whereas grade ≥3 elevation of asparatate aminotransferase, alanine aminotransferase and alkaline phosphatase was seen mainly in CG8, grade ≥3 non-hematologic toxicities such as febrile neutropenia, infection, appetite loss, diarrhea and eruption were observed only in CG1. ***Conclusion:*** CG1 is superior in response rate, but CG8 shows improved toxicities and a tendency of prolonged survival.


## Introduction

Cytotoxic chemotherapy still plays a pivotal role in the management of advanced non-small cell lung cancer (NSCLC), whereas recently, the combination of platinum doublets and molecular targeted drugs has been extensively studied. Among several platinum doublets, the combination of carboplatin and gemcitabine (CG) is a popular regimen. Carboplatin is commonly used in a practical setting compared with cisplatin, although the antitumor effect of carboplatin is suggested to be somewhat inferior to cisplatin [1–3]. Several studies have shown that platinum doublets with 3rd-generation cytotoxic drugs were similar in antitumor activities but different in toxicities [4]. A recent trial showed that pemetrexed/cis-

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platin produced better survival compared with gemcitabine/cisplatin in NSCLC with non-squamous (Non-Sq) histology [5]. In contrast, Grønberg et al. [6] showed that the superiority in survival of pemetrexed over gemcitabine is not clear when carboplatin was combined with these drugs. Hematologic toxicities of CG were more severe than those of pemetrexed/carboplatin: grade ≥3 leukopenia 46 and 23% ($p = 0.001$), neutropenia 51 and 40% ($p = 0.024$) and thrombocytopenia 56 and 24% ($p = 0.001$) in CG and pemetrexed/carboplatin, respectively. More patients in the CG arm received transfusions of red blood cells and platelets. If hematologic toxicities of CG regimens are improved, its application will be extended in the 1st-line treatment for Sq NSCLC or as an alternative of pemetrexed/carboplatin for Non-Sq NSCLC.

Clinical trials including CG regimens initially adopted a 4-week regimen. Subsequently, 3-week schedules in which gemcitabine was administered on days 1 and 8 were evaluated. In phase II studies, 3-week regimens showed comparable efficacy with the 4-week schedules [7]. In the 4-week arm, carboplatin at an area under the curve (AUC) of 5 mg/ml × min was administered on day 8 and gemcitabine 1,000 mg/m$^2$ was given on days 1 and 8. In the 3-week arm, carboplatin at AUC 5 was given on day 1, and gemcitabine at 1,000 mg/m$^2$ was administered on days 1 and 8. Obasaju et al. [8] compared 3- and 4-week schedules of CG in 472 advanced NSCLC patients. Although statistically not significant, 2% complete responses and 38% partial responses produced by the 3-week schedule are better than no complete response and 23% partial responses by the 4-week schedule. In contrast, the frequency of grade 3 or 4 thrombocytopenia, which is particularly problematic in CG, was 14% in the 3-week schedule and 8% in the 4-week schedule. A Japanese comparative phase II study, where 3-week schedule CG was compared with the combination of gemcitabine and vinorelbine, also showed a high incidence of dose reduction and early withdrawal due to myelosuppression, mainly thrombocytopenia, in CG [9].

These results indicate that hematologic toxicities of the 3-week CG regimen should be improved. We already reported a new regimen, in which gemcitabine of 1,000 mg/m$^2$ was administered on days 1 and 8 and carboplatin of AUC 5 on day 8 every 21 days. A schedule-dependent synergistic effect of gemcitabine combined with pemetrexed was reported in the treatment of NSCLC cell lines [10]. A similar schedule-dependent synergy may be observed in the CG combination. In a preceding phase II trial with 31 patients with stage IIIB or IV NSCLC, the response rate was 22.6%, including one complete response, and median time to progression and median survival time were encouraging, i.e. 161 and 454 days, respectively [11]. In this regimen, the criteria to start new cycles and those to perform day 8 infusion were specially defined in order to improve myelosuppression and to maintain dose intensity: white blood cell count (WBC) ≥2,500/mm$^3$ and platelet count (Plt) ≥750,000/mm$^3$ to start new cycles and WBC ≥3,000/mm$^3$ and Plt ≥100,000/mm$^3$ to perform day 8 infusion. As reported previously, these criteria were proved to work well, resulting in grade 3/4 thrombocytopenia only in 2 patients (6.5%; one grade 3 and one grade 4). Since this phase II study was performed in a single institution, we have compared the 3-week CG regimen that we developed with the standard 3-week CG regimen in a multi-institutional randomized phase II study.

## Patients and Methods

*Eligibility*

Patients were eligible for study participation when they met the following criteria: age <75 years; histologic or cytologic diagnosis of NSCLC; clinical stage IIIB not amenable to curative treatment or stage IV by the Union for International Cancer Control TNM classification version 6; first-line treatment; Eastern Cooperative Oncology Group performance status 0–1; measurable disease in Response Evaluation Criteria in Solid Tumors (RECIST); adequate bone marrow reserve (neutrophil count ≥1,500/mm$^3$, Plt ≥10 × 10$^4$/mm$^3$, hemoglobin ≥9.0 g/dl); acceptable hepatic (serum bilirubin <1.5 mg/dl, transaminases less than twice the upper limit of normal) and renal function (normal serum creatinine and creatinine clearance determined by Cockcroft equation ≥50 ml/min), and a life expectancy of at least 3 months. Patients were excluded from the study when they met one of the following conditions: active uncontrolled infection; unstable concomitant disease (ischemic heart disease, hypertension, arrhythmia, cirrhosis and diabetes mellitus); active concomitant malignant disease; massive effusion; concomitant interstitial lung disease; superior vena cava syndrome; brain metastasis, and pregnancy or breastfeeding. Written informed consent was obtained from all patients.

*Study Design*

The eligible patients were randomized to the CG1 or the CG8 arm. In the CG1 arm, carboplatin of AUC 5 calculated using the Calvert formula with creatinine clearance evaluation by the Cockcroft equation and gemcitabine of 1,000 mg/m$^2$ were administered as an intravenous injection on day 1 and on days 1 and 8, respectively (CG1). In the CG8 arm, the same doses of CG were administered as an intravenous injection on day 8 and on days 1 and 8, respectively (CG8). Treatment was repeated every 3 weeks. The two arms are similar, but developed independently. Therefore, we adopted independent hematologic criteria suitable for each arm to start new cycles and to perform day 8 infusion: in the CG1 arm, WBC ≥3,000/mm$^3$ and Plt ≥10 × 10$^4$/mm$^3$ were required to start new cycles, and WBC ≥2,000/mm$^3$ and Plt ≥100,000/mm$^3$ were necessary to perform day 8 infusion. In the CG8 arm, corresponding hematologic criteria were the same as

those in our preceding phase II study described in the Introduction section: WBC ≥2,500/mm$^3$ and Plt ≥750,000/mm$^3$ to start new cycles, and WBC ≥3,000/mm$^3$ and Plt ≥100,000/mm$^3$ to perform day 8 infusion. When one of these criteria was not met, treatment was skipped. A dose reduction of up to two times was permitted in the case of a leukocyte count <1,000/mm$^3$, Plt <25,000/mm$^3$, febrile neutropenia, grade >2 non-hematologic toxicity, or skip of day 8 administration in the preceding cycle. The dose of gemcitabine was reduced to 800 mg/m$^2$ in the first dose reduction and that of carboplatin to AUC 4 in the second one. After withdrawal from the study, subsequent treatment was decided by the investigator.

This study was performed by JACCRO (Japanese Cancer Clinical Research Organization) as an LC-01 study.

*Evaluation of Toxicity and Response*

Toxicity was scored every 3 weeks during treatment and every month thereafter according to the National Cancer Institute Common Toxicity Criteria version 2.0. Response was evaluated every 4 weeks during treatment and every 6 months until disease progression thereafter, according to RECIST criteria [12]. Brain MRI, chest CT scan and abdominal CT scan were performed at any time if assessment for disease progression was necessary. Objective responses were required to be confirmed after at least 4 weeks.

*Endpoint and Statistical Analysis*

The primary endpoint of this study was the response rate. Secondary endpoints included overall survival, toxicities, completion rate of 1–3 cycles, and dose intensity during 1–3 cycles. If the threshold response rate and the expected response rate were to be 20 and 35%, respectively, the study has 90% power to detect the better arm with 90% confidence using Simon's selection design when 29 patients were included in each arm. The Kaplan-Meier method was used to plot overall survival.

## Results

*Patient Characteristics*

From February 2005 to April 2007, 55 patients were enrolled in the study. Protocol amendment was done to prolong accrual time because of slow accrual, but the entry of the patients was finally stopped before completing the planned accrual of 60 patients. One patient was ineligible because of preceding chemotherapy, and 2 patients who had been allocated to the CG1 arm received CG8. Excluding these 3 patients, 25 patients in the CG1 arm and 27 patients in the CG8 arm were analyzed. Patient backgrounds are shown in table 1. Although ages, stages and histology are well balanced between the two arms, the ratios of men and the patients with a performance status of 1 seem to be slightly higher in the CG1 arm.

*Treatment Delivery*

The median number of the cycles administered was 3 in both arms. Whereas more patients underwent the 2nd to 4th cycle in the CG8 arm compared with the CG1 arm, the percentage of the patients who received the 5th and 6th cycle was higher in the CG1 arm. As shown in table 2, 52.0% of the patients in the CG1 arm and 66.7% in the CG8 arm received at least 3 cycles of chemotherapy, and 40.0% of the patients in the CG1 arm and 33.3% in the CG8 arm did without skips of administration schedule. Skipping administration on day 8 occurred in 36 and 48.1% in CG1 and CG8, and dose reduction was necessary in 56.0 and 40.7% in CG1 and CG8, respectively. Reflecting these modifications, the relative dose intensity of gemcitabine was 67.2% of the planned dose in CG1 and 74.4% in CG8, and that of carboplatin was 76.3% in CG1 and 68.1% in CG8 in the first 3 cycles (table 2).

**Table 1.** Patient characteristics

| | Arm A | Arm B |
|---|---|---|
| Patients | 25 | 27 |
| Age, years | | |
| Mean | 59.2 | 61.4 |
| Range | 40–74 | 40–73 |
| Men/women | 19/6 | 15/12 |
| Performance status 0/1 | 10/15 | 14/13 |
| Stage (IIIb/IV/after operation) | 5/18/2 | 4/21/2 |
| Histology (Ad/Sq/La) | 22/2/1 | 21/4/2 |

Ad = Adenocarcinoma; Sq = squamous cell carcinoma; La = large cell carcinoma.

**Table 2.** Treatment delivery and dose intensity

| | Arm A | Arm B |
|---|---|---|
| Completion of 3 cycles, % | | |
| Overall | 52.0 | 66.7 |
| Without skips | 40.0 | 33.3 |
| With skips | 12.0 | 33.3 |
| Dose intensity, % of planned dose | | |
| Carboplatin | 76.3 | 68.1 |
| Gemcitabine | 67.2 | 74.4 |

*Efficacy Results*

The response rate in the CG1 arm was 29.2% and is higher than that in the CG8 arm (22.2%; table 3). The response rates of both arms exceeded the threshold of 20%, but that of the better one did not reach the expected value of 35%. The rate of progression was higher in CG1 than

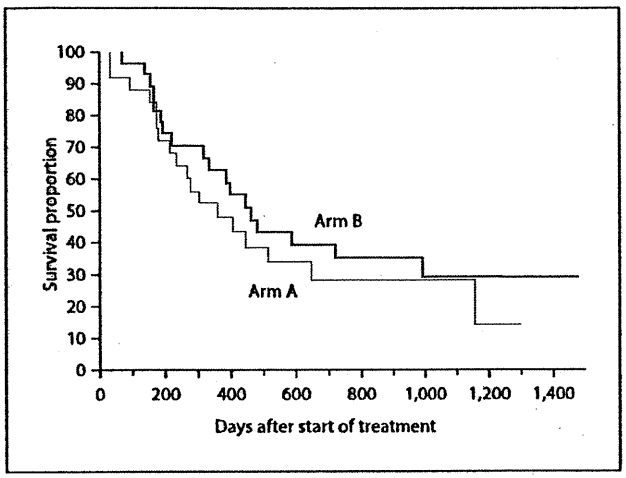


**Fig. 1.** Survival curves. Survival curves of arms A and B were drawn by the Kaplan-Meier method. The difference is not statistically significant (p values are 0.40 and 0.41 by the log-rank test and Wilcoxon test, respectively).

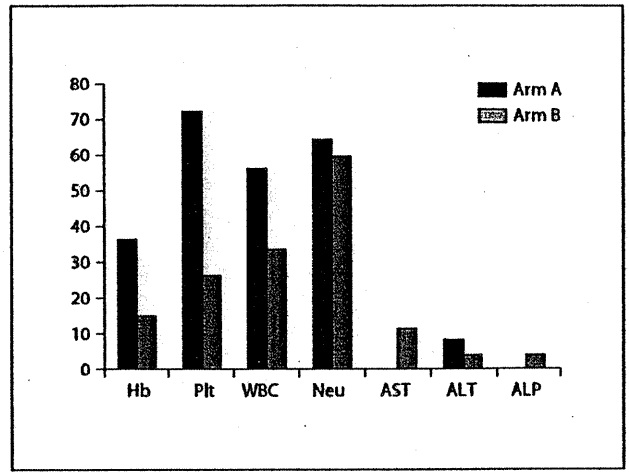


**Fig. 2.** Hematologic toxicity and abnormal blood chemistry of grade 3 and 4. Toxicities in blood hemoglobin value (Hb), WBC and neutrophil counts (Neu) are shown. AST = Asparatate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase.

**Table 3.** Response and survival

| | Arm A | Arm B |
|---|---|---|
| Response rate, % | 29.2 | 22.2 |
| CR | 4.0 | 0 |
| PR | 24.0 | 22.2 |
| SD | 40.0 | 51.8[1] |
| PD | 28.0 | 25.9 |
| NE | 4.0 | 0 |
| Median survival time[2], days | 348 | 455.5 |

CR = Complete response; PR = partial response; SD = stable disease; PD = progressive disease; NE = not evaluable.
[1] Including one unconfirmed partial response.
[2] p = 0.40 by the log-rank test; p = 0.41 by the Wilcoxon test.

that in CG8. Overall survival curves were shown in figure 1. Median overall survival time was 348 and 455.5 days in the CG1 and CG8 arm, respectively.

*Toxicity*

Toxicity profiles are summarized in figure 2 and table 4. Hematologic toxicities were generally milder in CG8 than in CG1: grade 3/4 leukopenia was observed in 56.0 and 33.3% of the patients, grade 3/4 thrombocytopenia in 72.0 and 25.9%, and grade 3/4 anemia (hemoglobin) in 36.0 and 14.8% in the CG1 and CG8 arm, respectively, whereas the frequency of grade 3/4 neutropenia was comparable between the two arms (64.0 and 59.2%, respectively). Grade 4 thrombocytopenia occurred in 4.0% in CG1, whereas no grade 4 thrombocytopenia was observed in CG8. Abnormal blood chemistry was sporadically observed but not severe in both arms. Table 4 summarizes non-hematologic toxicities of grade ≥3 except those in blood chemistry. Whereas the grade ≥3 elevation of asparatate aminotransferase, alanine aminotransferase and alkaline phosphatase was seen mainly in CG8, grade ≥3 non-hematologic toxicities such as febrile neutropenia, infection, appetite loss, diarrhea and eruption were observed only in CG1.

## Discussion

Recently, there has been progress in medical treatment for advanced NSCLC, incorporating molecular targeted drugs such as epidermal growth factor receptor tyrosine kinase inhibitors and bevacizumab, an angiogenesis inhibitor. However, cytotoxic drugs are still playing a pivotal role acting as themselves or as platforms with which molecular targeted drugs are combined. Less toxic chemotherapy regimens will have the advantages to maintain the quality of life of the patients

**Table 4.** Non-hematologic toxicities

| | Arm A, % | | Arm B, % | |
|---|---|---|---|---|
| | G2 | G3 | G2 | G3 |
| Febrile neutropenia | – | 4.0 | – | 0 |
| Infection | 0 | 8.0 | 0 | 0 |
| Body weight loss | 0 | 0 | 3.7 | 0 |
| Fever | 0 | 0 | 3.7 | 0 |
| General fatigue | 0 | 0 | 7.4 | 0 |
| Dyspnea | 8.0 | 0 | 3.7 | 0 |
| Nausea | 8.0 | 0 | 7.4 | 0 |
| Vomiting | 4.0 | 0 | 0 | 0 |
| Appetite loss | 0 | 4.0 | 0 | 0 |
| Diarrhea | 4.0 | 4.0 | 3.7 | 0 |
| Stomatitis | 0 | 0 | 3.7 | 0 |
| Constipation | 16.0 | 0 | 11.1 | 0 |
| Atrial fibrillation | 4.0 | 0 | 0 | 0 |
| Eruption | 12.0 | 4.0 | 7.4 | 0 |

G2 = Grade 2; G3 = grade 3. No grade 4 and 5 toxicities were observed. Adverse events were graded by the National Cancer Institute Common Toxicity Criteria version 2.0.

and to combine them with molecular targeted drugs. The CG combination was tested in various situations of NSCLC patients such as in an adjuvant setting, for the elderly and as a second line for selected patients, showing encouraging effects [13–15]. In addition, an acceptable toxicity and promising median overall survival was reported in the combination of CG and bevacizumab for Non-Sq NSCLC [16]. The management of carboplatin doublets is shown to be improved by introducing a clinical pathway [17].

We studied a 3-week CG regimen in which carboplatin is administered on day 8 and gemcitabine on days 1 and 8. In the present study, we compared two 3-week CG regimens, CG1 and CG8. The results show that the response rate, the primary endpoint of this study, is higher in CG1 than in CG8. The response rate in CG8 in our preceding phase II study described above was 22.6%, which was reproduced in this study. Since this study was conducted to select the better arm with regard to the response rate, one main conclusion of this study is that CG1 should be selected in subsequent studies.

In addition to the difference in response rate, our study also clarifies several useful differences between CG1 and CG8. In both hematologic and non-hematologic toxicities, CG8 is less toxic than CG1. Hematologic toxicities, especially thrombocytopenia, of CG8 are milder than those of CG1. Survival of the patients with CG8 is better than that of patients with CG1. Median survival time in the CG1 group is 348 days and is comparable to that of paclitaxel/carboplatin in the Four Arm Chemotherapy Study, a phase III study for advanced NSCLC performed in Japan [18]. Median survival time in the CG8 group is 455.5 days and is longer than that of CG1, although statistically not significant. The overall survival time of CG8 patients in our preceding phase II trial (454 days) was again reproduced. The difference in overall survival between CG1 and CG8 is hard to explain, because of the comparable dose intensity between the two arms and a better response rate in CG1. Reduced toxicity together with increased dose intensity of gemcitabine in CG8 may contribute to some extent to a possible prolonged survival. Increased dose intensity without deteriorating the patients' conditions may lead to prolonged survival and improvement in quality of life. Therefore, dose intensity could be selected as a primary endpoint in this study. The results show that dose intensities of both arms are comparable; the dose intensity of carboplatin is higher in CG1 and that of gemcitabine is higher in CG8. Progression-free survival time or overall survival time might have been a better endpoint. It may be possible to select tumor markers such as CYFRA21-1 or carcinoembryonic antigen as an endpoint in certain trials, because it is reported that they are valuable in evaluating chemotherapy in NSCLC [19].

CG was one of the least toxic platinum doublets. Recently, pemetrexed was introduced in the treatment of Non-Sq NSCLC. Pemetrexed/carboplatin produced comparable efficacy and showed statistically significant improvement in hematologic toxicities, when compared with CG1. Although pemetrexed/platinum is the first-choice regimen for Non-Sq NSCLC, CG is considered to be used for Sq NSCLC and even for Non-Sq NSCLC in an individual patient setting. In future chemotherapy for NSCLC, molecular targeted drugs will be used in combination with cytotoxic regimens. There are growing evidences showing that some molecular targeted drugs may require a suitable selection of cytotoxic regimens to exhibit cooperation [20–22]. Therefore, several kinds of platinum doublets, such as pemetrexed/platinum, paclitaxel/carboplatin and gemcitabine/platinum, should be tested in combination with new drugs. Our study may improve the usage of CG regimens, alone and in combination with molecular targeted drugs.

## Conclusion

Whereas a standard 3-week schedule of CG (CG1) should be selected in subsequent studies because of a higher response rate, the new schedule (CG8) seems less toxic and shows a tendency of better survival.


## Acknowledgment

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# Mesenchymal Stromal Cells Protect Cancer Cells From ROS-induced Apoptosis and Enhance the Warburg Effect by Secreting STC1

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Previous studies have demonstrated that mesenchymal stromal cells (MSCs) enhance cell survival through upregulation and secretion of stanniocalcin-1 (STC1). This study shows that MSC-derived STC1 promotes survival of lung cancer cells by uncoupling oxidative phosphorylation, reducing intracellular reactive oxygen species (ROS), and shifting metabolism towards a more glycolytic metabolic profile. MSC-derived STC1 upregulated uncoupling protein 2 (UCP2) in injured A549 cells in an STC1-dependent manner. Knockdown of UCP2 reduced the ability of MSCs and recombinant STC1 (rSTC1) to reduce cell death in the A549 population. rSTC1-treated A549 cells displayed decreased levels of ROS, mitochondrial membrane potential (MMP), and increased lactate production, all of which were dependent on the upregulation of UCP2. Our data suggest that MSCs can promote cell survival by regulating mitochondrial respiration via STC1.

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## INTRODUCTION

Mesenchymal stem or stromal cells (MSCs) reside in multiple organs, can be isolated and expanded for cell therapy, and have been shown to contribute to tissue repair by several mechanisms.[1] MSCs can home and contribute to the tumor stroma but there are conflicting reports as to whether the MSCs support or suppress tumor growth.[2,3] We previously observed that MSCs responded to signals from apoptotic cells by upregulation and secretion of stanniocalcin-1 (STC1).[4] STC1 is a evolutionarily conserved secreted protein that exerts pleiotropic effects including alteration of mitochondrial function by upregulation of uncoupling protein 2 (UCP2).[5–9] Here, we demonstrate that co-culture of MSCs with lung cancer cell lines made apoptotic by $H_2O_2$ or chemotherapeutic drugs activated MSCs to secrete STC1. The STC1 reduced apoptosis by upregulating UCP2 in the cancer cells to enhance the increased anaerobic glycolysis that is referred to as the Warburg effect and that promotes the growth of cancers. The results suggest that antibodies or antagonists to STC1 might counteract some of the effects of tumor stroma and provide a useful therapy for some cancers. The results also suggest that therapy using MSCs may itself be a double-edged sword.

## RESULTS

To test whether STC1 secreted by MSCs reduced ROS-induced cell death, we used cultures with A549 cells, a line of human alveolar basal epithelial adenocarcinoma cells. The A549 cells were made apoptotic by the addition of 100 μmol/l $H_2O_2$[10,11] and cultured alone or in the presence of MSCs grown on a transwell filter (outlined in **Supplementary Figure S1**). MSCs promoted the survival of A549 cells as measured by annexin V/propidium iodide (PI) staining and flow cytometry (Figure 1a), and by lactate dehydrogenase release (Figure 1b). STC1 transcripts and protein were upregulated in MSCs stimulated by $H_2O_2$ (Figure 1c). Blocking STC1 in the co-culture with anti-STC1 antibodies reduced the ability of the MSCs to promote cell survival (Figure 1d). Addition of recombinant STC1 (rSTC1) (12.5, 25, 50 ng/ml) was sufficient to increase survival of A549 cells exposed to $H_2O_2$ (Figure 1e). rSTC1 also promoted survival of A549 cells exposed to $H_2O_2$ as measured using a WST8 assay at a 48-hour timepoint (Figure 1f). To test longer term effects, increased the incubation time of the experiments. rSTC1 increased the survival of A549 cells exposed to $H_2O_2$ in experiments that were extended for 5 days (**Supplementary Figure S2**). Similar results were obtained with three additional lung cell lines, two additional lung epithelial adenocarcinoma (H1299 and PC9) and one lung epithelial squamous cell carcinoma (EBC1) lines. However, rSTC1 had no effect on the survival of squamous cancer cell line (LK2).

We next sought to determine whether the STC1-mediated increased survival of A549 cells could be attributed to decreased ROS production. Knockdown of STC1 in MSCs with short

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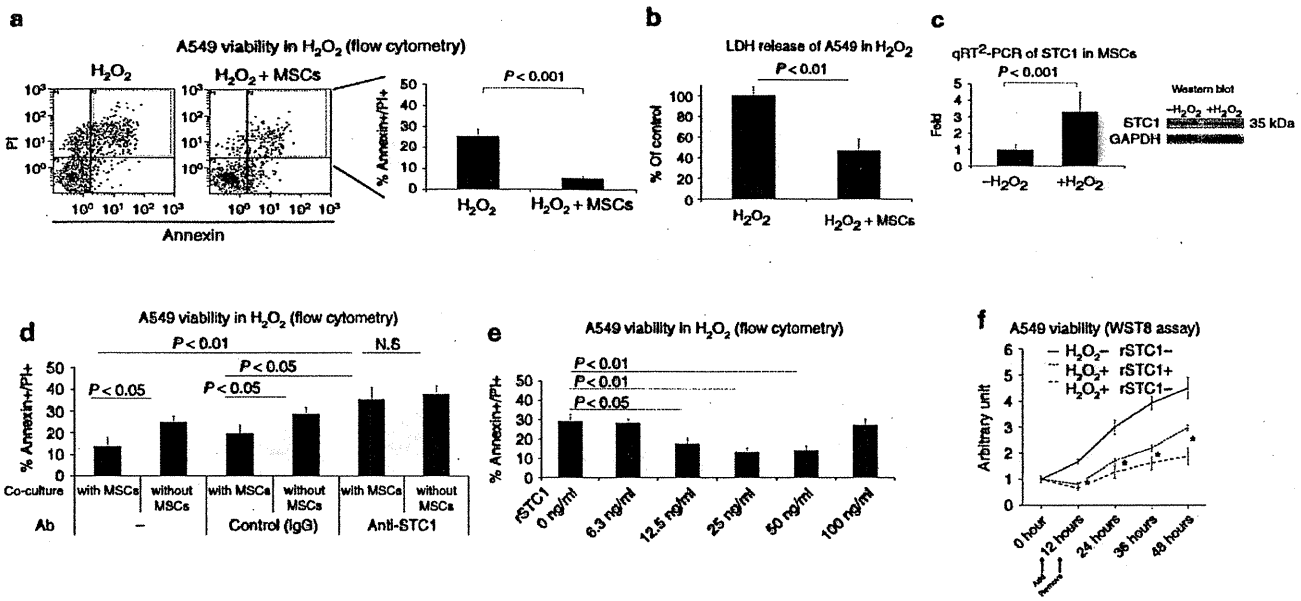


Figure 1 MSCs reduced ROS-induced cell death and cytotoxicity in A549s in a STC1-dependent manner. **(a)** A549 cells were incubated in a 6-well plate in culture medium containing $H_2O_2$ (100 µmol/l) with or without MSCs on a transwell filter (**Supplementary Figure S1**). Seven hours later, cell survival was assayed by flow cytometry for annexin-V staining and PI incorporation. **(b)** A549 cells were grown as in **a** and assayed for viability by LDH release. **(c)** STC1 expression in MSCs cultured in conditioned media with or without $H_2O_2$ (100 µmol/l) as evaluated using RT-PCR and western blot. **(d)** A549 cells were exposed to $H_2O_2$ (100 µmol/l) in the presence or absence of MSCs. Each culture was then incubated with control IgG or anti-STC1 antibodies and assayed for cell viability by flow cytometry. **(e)** Recombinant human STC1 was added to A549 cells exposed to $H_2O_2$ (100 µmol/l) and assayed by flow cytometry. **(f)** A549 cells were exposed to $H_2O_2$ (100 µmol/l) with or without rSTC1 and assayed every 12 hours up to 48 hours for viability by WST8 assay. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MSC, mesenchymal stromal cell; LDH, lactate dehydrogenase; PI, propidium iodide, ROS, reactive oxygen species; rSTC1, recombinant stanniocalcin-1; RT-PCR, reverse transcription-PCR.

interfering RNA (siRNA) (**Supplementary Figure S3**) inhibited cytoprotection of $H_2O_2$-injured A549 cells (Figure 2a). A549 cells grown alone or in the presence of MSCs were exposed to $H_2O_2$ for 4 hours and assayed by flow cytometry for ROS production. The co-cultures displayed a 30% reduction in ROS compared to A549 cells cultured alone (Figure 2b). Knockdown of STC1 in MSCs by siRNA resulted in increased ROS production in the A549 cells exposed to $H_2O_2$ compared to ROS production by A549 cells co-cultured with MSCs transduced with a nonspecific siRNA (Figure 2c). In addition, as expected, addition of rSTC1 decreased ROS in A549 cells cultured with $H_2O_2$ to control levels (Figure 2d). Addition of rSTC1 also reduced ROS in three other cell lines cultured in the presence of $H_2O_2$ (HL1299, PC9, ad EBC1 in **Supplementary Figure S4**). A fourth cell line (LK2) was unresponsive to rSTC1. Unexpectedly, STC1 reduced ROS in one cell line (PC9) incubated in the absence of $H_2O_2$ (**Supplementary Figure S4**). To expand our observations to a more clinically relevant model of ROS induction, we damaged A549 cells with the chemotherapeutic drug, paclitaxel.[12] Treatment of A549 cells with paclitaxel increased ROS production to the same extent as $H_2O_2$ (Figure 2e). Treatment of cells with the ROS scavenger N-acetyl cysteine, as well as STC1, reduced ROS production. To test whether $H_2O_2$ had any effect on the biochemical action of STC1, we exposed 500 µg/ml rSTC1 to 10 mmol/l $H_2O_2$ for 1 hour. $H_2O_2$-exposed STC1 was as effective at reducing ROS production as nonexposed rSTC1 (Figure 2e, last column).

Previously, STC1 was shown to induce UCP2 expression in macrophages and thereby decrease ROS production.[7] To confirm whether STC1 increased UCP2 in A549 cells cultured with $H_2O_2$, rSTC1 was added to the culture medium and UCP2 expression was assayed in the A549 cells by real-time PCR and western blot. The rSTC1 increased expression of UCP2 (Figure 3a). We next tested whether STC1 produced by A549 cells contributed to the UCP2 production in the same cells incubated with $H_2O_2$. As expected, knockdown of STC1 in the A549 cells with a specific siRNA decreased the levels of messenger RNA for UCP2 (Figure 3b). The expression of UCP2 was also upregulated in A549s when co-cultured with MSCs and addition of anti-STC1 antibodies reduced this effect (Figure 3c).

To confirm that upregulation of UCP2 was the primary mechanism for the reduction of ROS, UCP2 in the A549 cells was knocked down with a specific siRNA. Downregulation of UCP2 in A549 cells was confirmed by real-time PCR and western blot (Figure 4a). Knockdown of UCP2 in A549 cells abolished the ability of rSTC1 to increase viability of A549 cells injured with $H_2O_2$ (Figure 4b). Also, knockdown of UCP2 by siRNA impaired the ability of rSTC1 to decrease ROS compared to the controls (Figure 4c). In order to corroborate the role of UCP2, we next assayed whether rSTC1 affected traditional pathways for ROS resistance in A549 cells. rSTC1had no effect on catalase, glutathione peroxidase or thioredoxin reductase (**Supplementary Figure S5**). These results suggested ROS reduction induced by rSTC1 was not dependent on these enzyme reactions but rather the upregulation of UCP2 in A549 cells.

We next tested the effects of STC1 on mitochondrial membrane potential (MMP) as assayed by flow cytometry using JC1

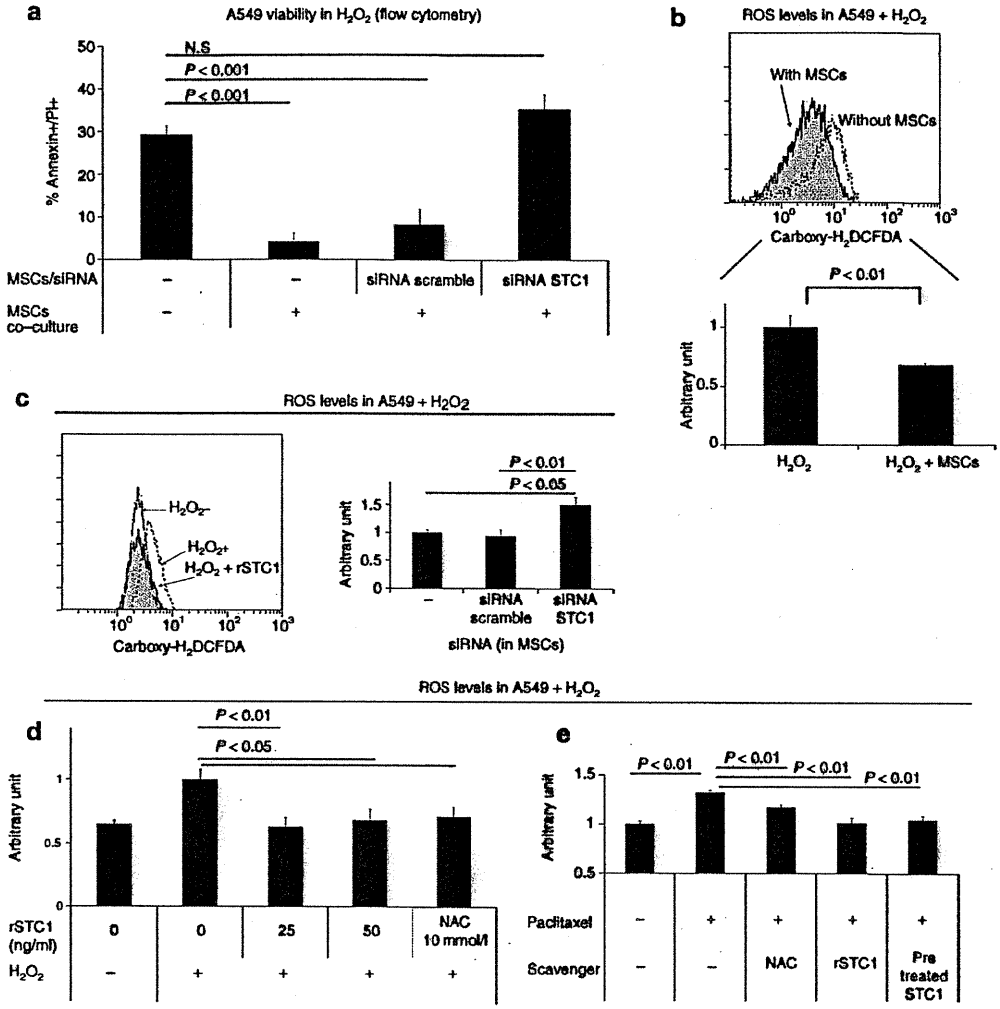


Figure 2 MSCs decreased ROS in $H_2O_2$-exposed A549 cells in a STC1-dependent manner. **(a)** A549 cells were incubated in culture medium containing $H_2O_2$ (100 µmol/l) with or without MSCs on a transwell filter. MSCs were transfected with a control or STC1 directed siRNA. Cells were assayed for viability using annexin V/PI staining. **(b)** A549 cells were incubated in a 6-well plate in culture medium containing $H_2O_2$ (100 µmol/l) with or without MSCs on a transwell filter. Four hours later, each culture was assayed for ROS using the dye, carboxy-H2DCFDA. Upper panel, representative flow cytometry data. Lower panel, quantification of ROS from three experiments. **(c)** MSCs transfected with scrambled or STC1-specific siRNA were co-cultured with A549 in medium containing $H_2O_2$ (100 µmol/l). Left panel: A549 cells were assayed for ROS using carboxy-H2DCFDA. Right panel: siRNA knockdown of STC1 in MSCs measured by real-time PCR. **(d)** rSTC1 was added to the A549 cell culture medium containing $H_2O_2$ (100 µmol/l). After 4 hours, A549 cells were assayed for ROS using carboxy-H2DCFDA. **(e)** NAC (N-acetylcysteine, 10 mmol/l), rSTC1 (50 ng/ml), and $H_2O_2$-pretreated rSTC1 (50 ng/ml) were added to A549 cells exposed to paclitaxel (5 µmol/l). Cells were assayed for ROS using carboxy-H2DCFDA. MSC, mesenchymal stromal cell; PI, propidium iodide; ROS, reactive oxygen species; siRNA, short interfering RNA; rSTC1, recombinant stanniocalcin-1.

dye. rSTC1 decreased MMP of A549 cells incubated under control conditions (Figure 5a), incubated with $H_2O_2$ (Figure 5a), or incubated under conditions of hypoxia and acidosis (**Supplementary Figure S6**). In contrast, rSTC1 increased MMP in A549 cells in which UCP2 was knocked down with a siRNA and the cells were exposed to $H_2O_2$ (Figure 5b). As expected, rSTC1 had no effect on MMP in the presence of a blocking antibody to STC1 (Figure 5c). Two additional lung epithelial adenocarcinoma (H1299 and PC9) and one lung epithelial squamous cell carcinoma (EBC1) lines behaved similarly when assayed for MMP with JC1 dye following incubation with $H_2O_2$ and rSTC1 (**Supplementary Figure S7**). One squamous cancer cell line (LK2) did not respond to rSTC1. The conditioned media of A549 cells was then assayed for the presence of lactate that is produced by anaerobic glycolysis. rSTC1 induced a 33% increase in lactate production in the presence or absence of $H_2O_2$ (Figure 5d). The increase in anaerobic glycolysis (about 0.4 pmol of lactate per cell equivalent to 0.2 pmol glucose) was not sufficient to be detectable by an increase in glucose utilization under the same conditions (Figure 5e).

To determine whether uncoupling of oxidative phosphorylation by STC1 increased nonphosphorylating respiration in A549 cells, we measured metabolic flux using an automated instrument (Seahorse Extracellular Flux Analyzer; Seahorse Bioscience, North

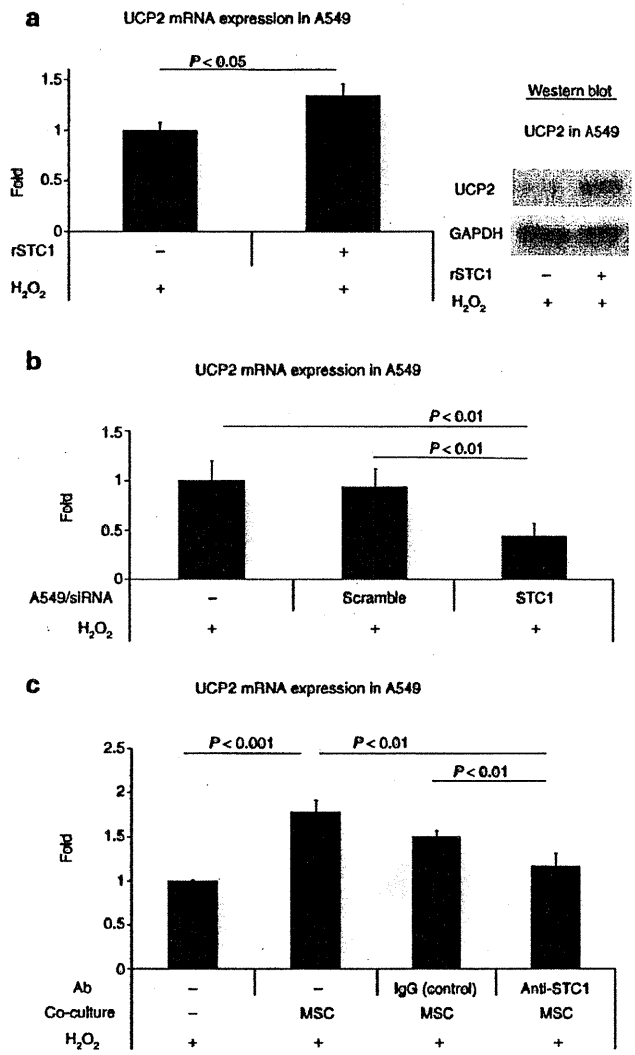


Figure 3 Autocrine and paracrine upregulation of UCP2 by STC1. (**a**) A549 cells were exposed to rSTC1 (50ng/ml) for 4 hours and assayed for UCP2 expression by real-time PCR (left panel) and western blot (right panel). (**b**) A549 cells cultured in medium containing $H_2O_2$ (100μmol/l) were assayed for UCP2 expression by real-time PCR following knockdown of STC1 by siRNA. (**c**) A549 cells were cultured in medium containing $H_2O_2$ (100μmol/l) with/without MSCs. Antibody vehicle only, control IgG, or anti-STC1 antibodies (500ng/ml) were added to the co-cultured A549 cells. MSC, mesenchymal stromal cell; siRNA, short interfering RNA; STC1, stanniocalcin-1; UCP2, uncoupling protein 2.

Billerica, MA), and using a Clarke electrode to measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) over time following injection of test compounds. To validate the adequacy of the measurements of OCR and ECAR, control experiments were performed with 2-deoxyglucose (2-DG) to stimulate aerobic metabolism (increased OCR, decreased ECAR), rotenone to promote anaerobic metabolism (decreased OCR, increased ECAR), and 2,4-dinitrophenol (2,4-DNP) to uncouple oxidative phosphorylation (increased OCR, increased ECAR). The expected responses were observed (Figure 5f, left panel). Treatment of the A549 cells with rSTC1 produced an increase in OCR and ECAR characteristic of uncoupling of oxidative phosphorylation, whereas control cells respired via aerobic metabolism (Figure 5f, right panel). Raw data of OCR and ECAR over 12 hours are displayed in Figure 5g. To confirm these data and investigate the effect of rSTC1 in $H_2O_2$-exposed cells, A549 cells were treated with rSTC1 in the absence or presence of $H_2O_2$ and oxygen consumption was measured using a Clarke electrode system. As expected, treatment of A549 cells with rSTC1 in the absence or presence of $H_2O_2$ led to an increase in OCR (Figure 5).

## DISCUSSION

The microenvironment provided by the stromas of tumors is a key determinant for the propagation and metastases of cancer cells.[13–15] The stromas can arise from a variety of cells, including bone marrow MSCs, tissue resident MSCs or even endothelial cells that can transform into MSC-like cells.[16–18] Previous reports demonstrated that co-culture of MSCs with leukemia cells increased the cancer-characteristic Warburg effect of excessive anaerobic glycolysis by upregulating the expression of UCP2 in the leukemia cells.[19,20] By uncoupling oxidative phosphorylation, UCP2 increases the efficiency of mitochondria in generating electrons to reduce ROS.[7,21] UCP2 can thereby contribute to the aberrant redox system of many cancer cells that allows them to proliferate in the presence of carcinogenic environments that generate ROS.[22] The stromas of tumors provide a large number of factors that enhance propagation and metastases of tumors and that probably vary with the stromas of different cancers.[23] The results here suggest that STC1 secreted by MSC-like cells in tumor stroma plays a critical role in enhancing the Warburg effect and making tumors resistant to ROS. Therefore blocking antibodies or antagonists to STC1 may be attractive candidates as an adjunct therapy for γ-radiation and other therapies that increase ROS. The results also highlight additional considerations that need to be addressed in order to fully understand the use of MSCs as a cell therapy. By understanding the processes that make MSCs a "double-edged sword"[22] in the context of some disease states, we may be able to modify the cells or culture conditions such that the therapeutic outcomes are more predictable.

## MATERIALS AND METHODS

For details, see Supplementary Materials and Methods.

***Cell culture and reagents.*** Frozen vials of passage 1 human bone marrow MSCs from three different donors were obtained from Tulane University (http://www.som.tulane.edu/gene_therapy/distribute.shtml; currently http://medicine.tamhsc.edu/irm/msc-distribution.html). For STC1 blocking and western blot assays, goat anti-STC1 antibodies were used as reported in our previous studies[4,5] (R&D Systems, Minneapolis, MN).

***Viability assay.*** Viability was measured by two independent methods, annexin V/PI staining and lactate dehydrogenase release assay. Cell proliferation was measured using 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-8 assay).

***Measurement of ROS.*** ROS were measured using acetoxymethyl ester dye, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, according to the manufacturer's instructions (C2938; Molecular Probes, Eugene, OR).

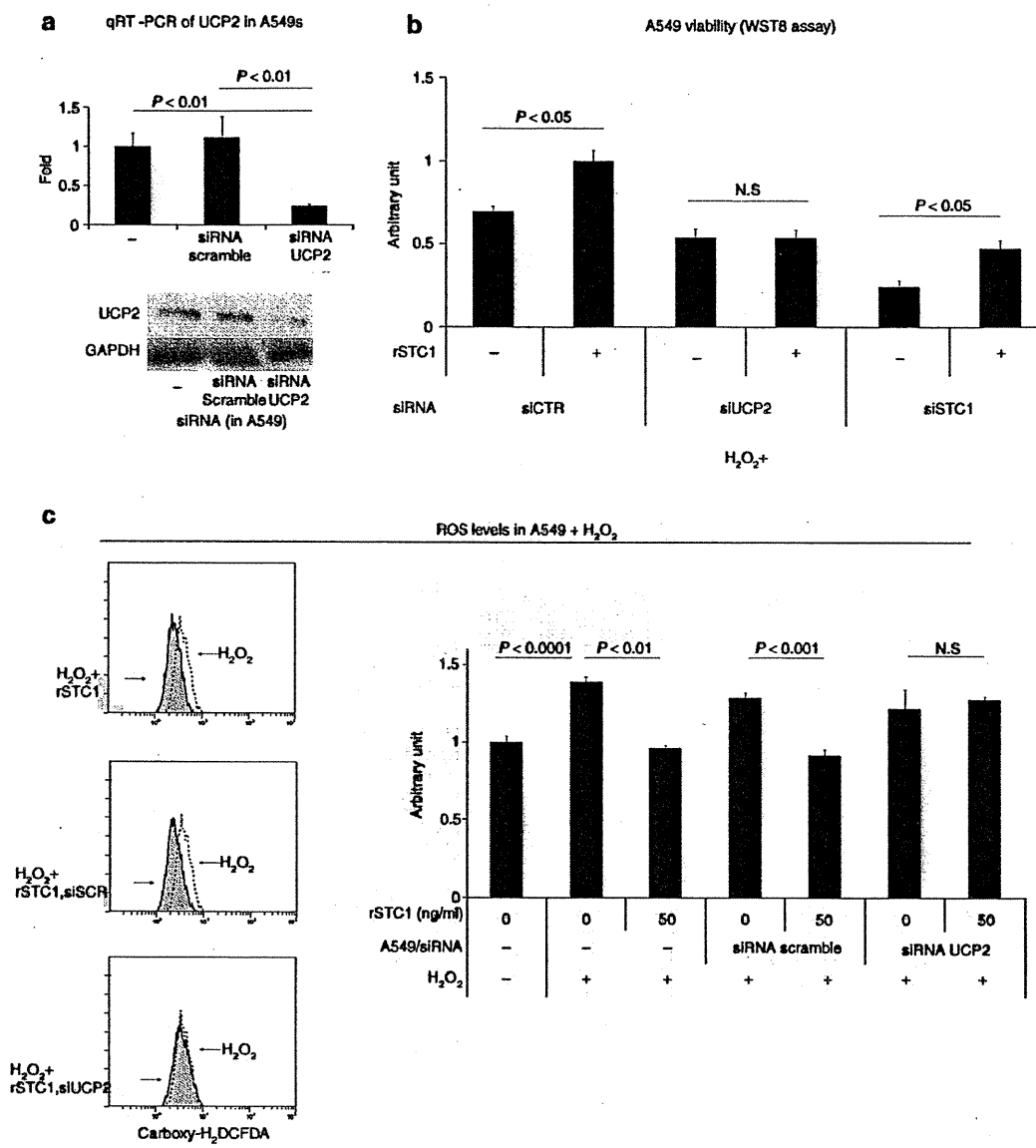


Figure 4 STC1-mediated reduction of ROS is dependent on UCP2. **(a)** A549 cells were transfected with siRNA-targeting UCP2 and assayed for UCP2 by real-time PCR (upper panel) and western blot assays (lower panel). **(b)** A549 cells were cultured in $H_2O_2$ (100 µmol/l) and transfected with control, UCP2 or STC1 directed siRNA. For each condition, cells were tested for the ability of rSTC1 to promote viability. **(c)** A549 cells were transfected with siRNA-targeting UCP2 or a scrambled siRNA control (siSCR) and cultured in medium containing $H_2O_2$ (100 µmol/l) and rSTC1 (50 ng/ml). After 4 hours, cells were assayed for ROS using carboxy-H2DCFDA. Left panel: representative flow cytometry images. Right panel: quantification of flow cytometry data from multiple experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative reverse transcription-PCR; ROS, reactive oxygen species; siRNA, short interfering RNA; STC1, stanniocalcin-1; UCP2, uncoupling protein 2.

***Measurement of MMP.*** MMP was measured using JC1 dye, according to the manufacturer's instructions (Molecular Probes).

***Lactate, glucose, catalase, glutathione peroxidase, and thioredoxin reductase activity measurements.*** Lactate and glucose concentrations as well as catalase, glutathione peroxidase, and thioredoxin reductase activity in the culture medium were measured using commercial colorimetric assays (BioVision, Mountain View, CA) and a microplate reader.

***Oxygen consumption measurements.*** OCRs were measured using an S1 Clarke electrode disc (Hansatech, Norfolk, UK).

***XF-24 OCR and ECAR measurements.*** OCR and ECAR were measured using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) according to manufacturer's instructions and as reviewed previously.[24]

***Statistical analyses.*** All experiments were performed a minimum of three times in triplicate. Analysis of variance followed by a Tukey's *post-hoc* test was performed for experiments with more than two groups; otherwise, a two-tailed, unpaired Student's *t*-test was performed. Statistical analyses were performed using Smith's Statistical Package (http://www.economics.pomona.edu/StatSite/SSP.html).

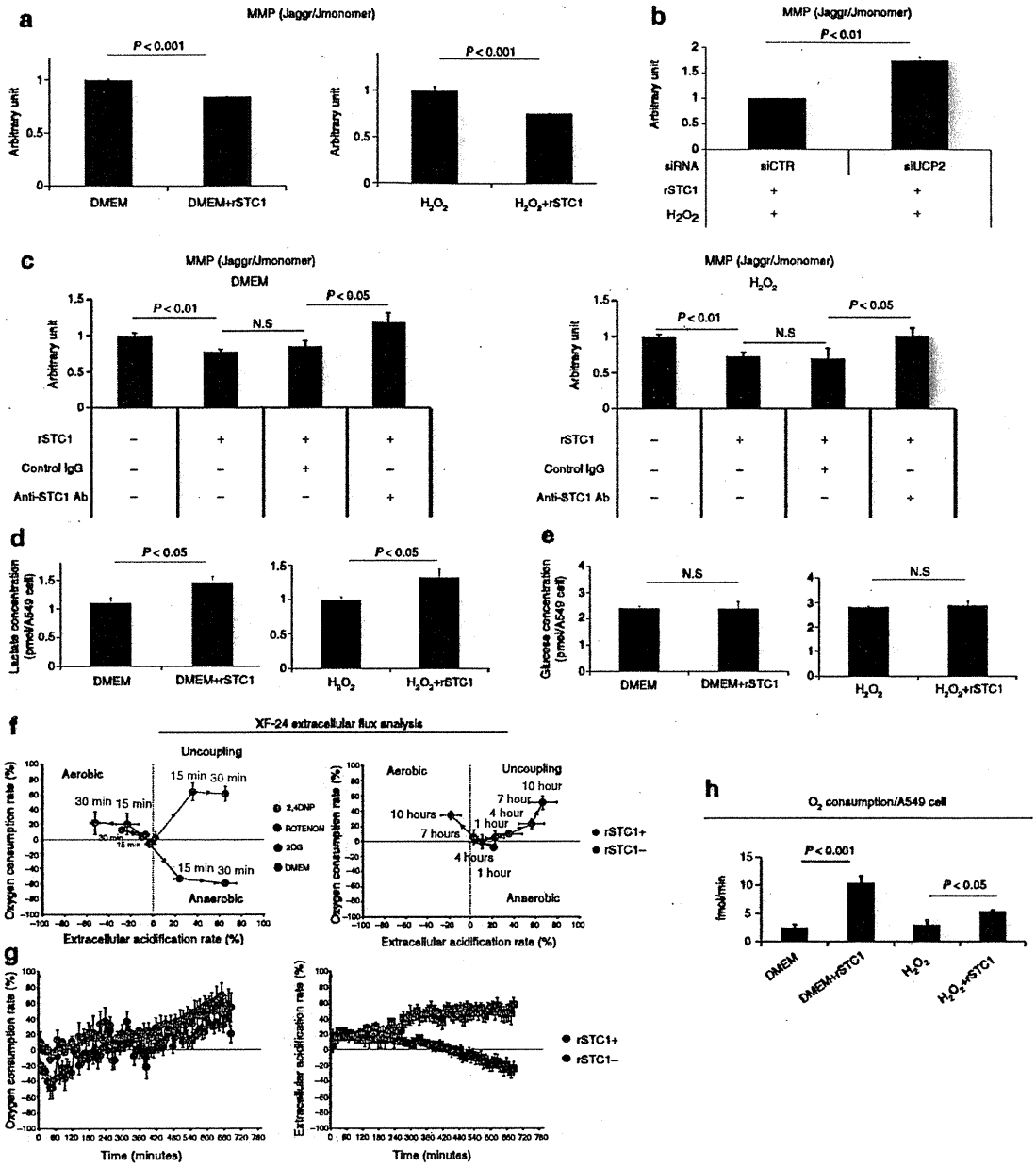


Figure 5 STC1 promotes anaerobic glycolysis in A549 cells. **(a)** A549 cells were cultured with or without $H_2O_2$ (100 µmol/l) and rSTC1 (50 ng/ml). Four hours later, cells were assayed for MMP by JC1 dye incorporation. Left panels, representative flow cytometry data. Right panels, quantification of MMP from multiple experiments. **(b)** A549 cells were cultured in $H_2O_2$ (100 µmol/l) and transfected with control (siCTR) or siRNA directed towards UCP2. Cells were assayed for MMP by JC1 dye incorporation. **(c)** A549 cells were cultured in the absence (left panel) or presence (right panel) of $H_2O_2$. Cells were treated with nonspecific IgG or anti-STC1 antibodies and assayed for MMP by JC1 dye incorporation. **(d)** A549 cells were cultured with or without $H_2O_2$ (100 µmol/l) and rSTC1 (50 ng/ml). Four hours later, cells were assayed for lactate production using a colorimetric assay. **(e)** A549 cells were cultured with or without $H_2O_2$ (100 µmol/l) and rSTC1 (50 ng/ml). Four hours later, conditioned medium was assayed for glucose levels using a colorimetric assay. As indicated in text, the increase in anaerobic glycolysis was detected by assays of lactate production but too small to detect by assay of glucose. **(f)** A549 cells were cultured on XF24-well plates to allow injection of compounds and subsequent detection of OCR and ECAR by the Seahorse XF Extracellular Flux Analyzer. Left panel: validation of the system. Cells cultured in DMEM were transferred to assay medium, exposed to test compounds, and then analyzed for OCR and ECAR for 30 minutes with 2-deoxyglucose (2-DG) to stimulate aerobic metabolism (aerobic), rotenone (ROTENON) to inhibit mitochondrial metabolism (anaerobic), and 2,4-dinitrophenol (2,4-DNP) to uncouple oxidative phosphorylation (uncoupling). Right panel: cells cultured in similar conditions were exposed to DMEM with or without rSTC1 (final concentration; 50 ng/ml) for 10 hours. **(g)** A549 cells were cultured as in **a**, and analyzed by the Seahorse system for 12 hours following injection of culture medium with or without rSTC1 (50 ng/ml). **(h)** A549 cells were cultured with or without $H_2O_2$ (100 µmol/l) and rSTC1 (50 ng/ml), and oxygen consumption rate was measured over time using a Clarke electrode. DMEM, Dulbecco's modified Eagle medium, ECAR, extracellular acidification rate; MMP, mitochondrial membrane potential; OCR, oxygen consumption rate; rSTC1, recombinant stanniocalcin-1; UCP2, uncoupling protein 2.

## SUPPLEMENTARY MATERIAL

**Figure S1.** Setup of the co-culture experiments.
**Figure S2.** rSTC1 promotes survival in other lung epithelial cell lines after 5 days.
**Figure S3.** Knockdown of STC1 in MSCs by siRNA.
**Figure S4.** Other lung epithelial cells also reduce ROS in response to rSTC1.
**Figure S5.** rSTC1 does not affect the activity of other canonical anti-oxidant enzymes.
**Figure S6.** rSTC1 reduces MMP in A549 cells grown in hypoxia or acidosis.
**Figure S7.** rSTC1 reduced MMP in other lung epithelial cell lines.
**Materials and Methods.**

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**Supplemental Informations;**

**Supplemental Materials and Methods**

**Cell culture and reagents shown in supplemental information.**

H1299 was purchased from ATCC (Rockville, MD), and PC9 from IBL (Gumma, Japan). EBC1 and LK2, were purchased from RIKEN (Tsukuba, Japan).H1299, PC9 and LK2 were cultured in RPMI 1640 Medium (Sigma-Aldrich) containing 10% FBS. A549 and EBC1 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco/BRL) containing 10% FBS.For the acidosis assay, the culture medium of A549 was preadjusted to a predetermined pH 6.3 with lactic acid (Sigma-Aldrich, St Louis, MO). For hypoxia experiments, the A549 cells were incubated under 1% $O_2$, 5% $CO_2$, and 94% $N_2$ for 24 hours (ACI-165 multigas incubator; ASTEC, Fukuoka, Japan).

**Primers for quantitive RT-PCR.**

STC-1 Forward: CAG CTG CCC AAT CACTTC TC; STC-1 Reverse: TCT CCA TCA GGCTGT CTC TGA;UCP-2Forward:ACA GTG TGG GAG GCC TGG TA; UCP-2 Reverse: AGG ACG AAG ATT CTG GCT GA; GAPDH Forward: TCA ACGGAT TTG GTC GTA TTG GG; and GAPDHReverse: TGA TTT TGG AGG GAT CTC GC. The adequacy of GAPDH as an internal control was confirmed by another internal control of 18s rRNAthat gave the same results in preliminary experiments (data not shown).