

Cyclin G1 overcomes radiation-induced G2 arrest and increases cell death through transcriptional activation of cyclin B1

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Abstract

Although cyclin G1 has been implicated in certain p53-related biological phenomena, other aspects of its function remain unclear. Here we report hitherto unknown mechanism by which cyclin G1 increases radiation sensitivity by regulating the level of cyclin B1. Overexpression of cyclin G1 was observable in lung carcinoma tissues. Irradiation of human lung cells with cyclin G1 overexpression resulted in increased cell death and γ -H2AX foci suggesting that cyclin G1 rendered the cells more susceptible to DNA damage. Enhanced radiosensitivity by cyclin G1 was correlated with increased cyclin B1, CDC2/cyclin B1 complex, and MPM2. Cell cycle synchronization clearly showed coexpression of cyclin G1 and cyclin B1 in G2/M phase. Depletion of cyclin G1 by interference RNA revealed that cyclin G1 regulated transcription of cyclin B1 in a p53-independent manner, and confirmed that the increased mitotic cells and cell death by cyclin G1 were dependent upon cyclin B1. Therefore, our data suggest that cyclin G1 enhanced radiation sensitivity by overriding radiation-induced G2 arrest through transcriptional upregulation of cyclin B1.

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Abbreviations: CDK5, cyclin-dependent kinase 5; GAK, cyclin G associated kinase; siRNA, interference RNAs

Introduction

Cyclin G1, which is one of the target genes of p53, has been investigated for its roles relevant to p53 activities.^{1,2} However, recent observations suggest that cyclin G1 might be involved in various p53-independent activities. Cyclin G1 is associated with cyclin-dependent kinase 5 (CDK5) and non-CDK-serine/threonine kinase, named cyclin G associated kinase (GAK).^{3,4} In addition, cyclin G1 has been suggested to act as an oncogenic protein, because of its overexpression in human tumor cells.^{5,6} On the other hand, cyclin G is also involved in G2/M arrest in response to DNA damage⁷ or in the facilitation of TNF-induced apoptosis,⁸ implicating tumor suppressive function. Despite these findings, understanding of the biochemical function of cyclin G1 in complex with associated partners and of the molecular function of association partners in cellular physiology has been limiting.

In response to IR cells induce checkpoints at G1/S, S, and G2/M phases as well as cell death or apoptosis.⁹

Within the cell cycle, both G1–S and G2–M phase transitions are under constant surveillance for the protection of cells from exogenous and endogenous DNA-damaging agents. These ordered dependencies are controlled by the regulation of certain gene products, whose mutations can result in altered stress responses, increased mutation rates, or genetic instability.¹⁰ Exposure to DNA-damaging agents, such as ionizing radiation, results in cell cycle arrest, thereby allowing the damaged chromosomes to be repaired before the cell progresses to mitosis. Therefore, investigation of the genetic mechanisms of cell cycle checkpoints is highly expected to contribute to an understanding of both cancer development and responses of cells to chemotherapy and radiotherapy. Progression from the G2 phase into mitosis requires the following: the binding of cyclin B1 to CDC2, a series of phosphorylation and dephosphorylation events on both of these subunits, and translocation of the CDC2–cyclin B1 complex to the nucleus.¹¹ Before mitosis, active CDC2 is held in an inactive state by phosphorylation^{12,13} and, at the end of G2, rapid dephosphorylation of CDC2 at Thr-14 and Tyr-15 results in entry into mitosis.^{14,15} The rapid dephosphorylation of CDC2 probably involves simultaneous inactivation of the Wee1/Myt 1 kinases together with the activation of the dual specificity CDC25C phosphatase, which in turn dephosphorylates CDC2 at Thr-14, followed by Tyr-15.¹⁶

In our previous study, radiation response genes were identified from the peripheral blood lymphocytes by using microarray system. One of the genes, cyclin G1 was correlated by 92% with radiation responsiveness in *ex vivo* irradiated human lymphocytes.¹⁷ In order to investigate molecular mechanism how cyclin G1 affects radiation response, we examined (1) cyclin G1 expression in cancer tissues; (2) effects of cyclin G1 overexpression on cell cycle and (3) G2

arrest defect by cyclin G1. Cyclin G1 was relatively overexpressed in several cancer tissues, including lung as assessed by tissue array system. Cyclin G1 overexpression in lung carcinoma or fibroblast cells overcame radiation-induced G2 arrest by transcriptionally activating cyclin B1 and this in turn resulted in increased radiation sensitivity.

Results

Cyclin G1 overexpression facilitated radiation induced growth inhibition and cell death

Cyclin G1 has frequently been reported to be overexpressed in cancer tissues.^{5,6,18} Our immunocytochemical analysis by using tissue array revealed that tissues of lung carcinoma (Figure 1a), astrocytoma, melanoma, thyroid gland carcinoma, breast carcinoma, liver carcinoma, and cervix cancer (data not shown) overexpressed cyclin G1 protein. To examine the response to radiation in cyclin G1 overexpressing cells, cyclin G1 was transfected to lung cells such as NCI-H460 and WI38 cells by using retroviral vector system (Figure 1b). Clonogenic survival of both NCI-H460 and WI38 cells was inhibited by cyclin G1 overexpression. Radiation-induced growth delay was also facilitated by cyclin G1 overexpression, when Trypan blue dye exclusion assay was used (Figure 1c). At 48 h after 5 Gy radiation, cell death shown by PI staining was significantly increased (Figure 1d). Since the presence and the rate of loss of histone H2AX foci are correlated with cellular radiosensitivity,¹⁹ phosphorylated H2AX on serine 139 was examined by Western blotting analysis: Because phosphorylated H2AX can be visualized as foci by immunofluorescence using phosphospecific antibodies, foci formation was also examined. Phosphorylated H2AX was increased at 24 and 48 h after radiation with further potentiation by cyclin G1. Figure 1e and f shows that radiation induced H2AX foci were significantly increased by cyclin G1 overexpression.

Cyclin G1 overexpression inhibited radiation-induced G2 arrest

To elucidate the mechanisms of cyclin G1-mediated increased radio-sensitivity, cell cycle distribution was examined after radiation. As shown in Figure 2a, radiation-induced G2/M arrest was increased by cyclin G1 overexpression in both NCI-H460 and WI38 cells: 12 h of radiation induced 40% G2/M arrest in control cells and 64% G2/M arrest in cyclin G1 overexpressing NCI-H460 cells. At 24 h after radiation, 30% G2/M in the control and 52% in the cyclin G1 overexpressing cells. Similar pattern was also observed in the case of WI38 cells. To elucidate whether cyclin G1 affected G2 or M phase,²⁰ morphological analysis after Giemsa staining was performed. Radiation-induced mitotic index was significantly increased by cyclin G1 in both NCI-H460 and WI38 cells (Figure 2b). In addition, MPM-2 protein, which is known to be expressed in mitotic cells, was more predominantly expressed in cyclin G1 overexpressing NCI-H460 cells and WI38 cells (Figure 2c). When examined by Hoechst 33258 staining at 24 and 48 h after radiation (data of 24 h are not shown), multinuclear and giant cells were significantly increased in

both the cyclin G1 overexpressing cells (Figure 2d). When cell cycle regulators, which are involved in G2-M transition, were examined, basal levels of cyclin B1 and its counterpart kinase, CDC2 activity were increased in cyclin G1 overexpressing NCI-H460 and WI38 cells, whereas the expression of inactive phospho-CDC2 was decreased. The expressions of broad range of CDK inhibitors, p21 were decreased and cyclin B1 bound p21 proteins were also inhibited in cyclin G1 overexpressing cells, suggesting that cyclin G1 facilitated protein activities, which are involved in G2-M transition (Figure 3a). When the cells were irradiated, these phenomena were more predominant. Radiation-induced p21 expressions were attenuated by cyclin G1 overexpression in NCI-H460 and WI38 cells, whereas radiation-induced cyclin B1 expression was potentiated. Increase of CDC2 kinase activity and CDC2–cyclin B1 complex in response to radiation, which was accompanied with lower expression of phospho-CDC2 protein, was shown in cyclin G1 overexpressing cells, suggesting that cyclin G1 overexpression overcame radiation-induced G2 arrest in cell cycle regulation (Figure 3b). The expression levels of p53 were not different between the control and the cyclin G1 overexpressing cells with or without radiation.

Cyclin G1 was predominantly expressed in G2/M phase in cell cycle progression

Since cyclin G1 regulates G2–M transition, it appears to be quite logical to speculate that cyclin G1 expression correlates with cell cycle distribution. After 200 μ M mimosine treatment, which synchronizes cells at G1 phase, the amount of mitotic cells peaked at 12 h. This mitotic index coincided with the peak of G2/M phase with further augmentation by cyclin G1 overexpression (Figure 4a). Timing of cyclin G1 and cyclin B1 expression also overlapped with maximum at 12 h, when G2/M phase peaked. The expression pattern disappeared afterward as cell cycle transitioned from G2/M to G1 phase (Figure 4b). Immunofluorescence data of cyclin G1 overexpressed cells also indicated that cyclin G1 and cyclin B1 was maximum at 12 h after mimosine wash-out, when G2/M phase peaked.

Cyclin G1 regulated cyclin B1 expression in a p53-independent manner

Since cyclin G1 overexpression increased cyclin B1 expression and their expression was correlated with each other, we next examined whether cyclin G1 controlled cyclin B1 expression, by using interference RNAs (siRNA) for cyclin B1 and cyclin G1 in NCI-H460 and WI38 cells. As seen in Figure 3, siRNA of cyclin G1 (siG1) efficiently depleted cyclin G1 protein level in dose-dependent manner, whereas control siRNA (siCont) treatment did not. Interestingly, siG1 diminished cyclin B1 protein level whereas cyclin B1 inhibition with cyclin B1 siRNA (siB1) did not affect cyclin G1 expression (Figure 5a), suggesting that cyclin G1 is an upstream molecule for cyclin B1 expression. Since cyclin G1 has been reported to be regulated by p53,^{7,21} we examined whether cyclin B1 regulation by cyclin G1 was dependent on p53.

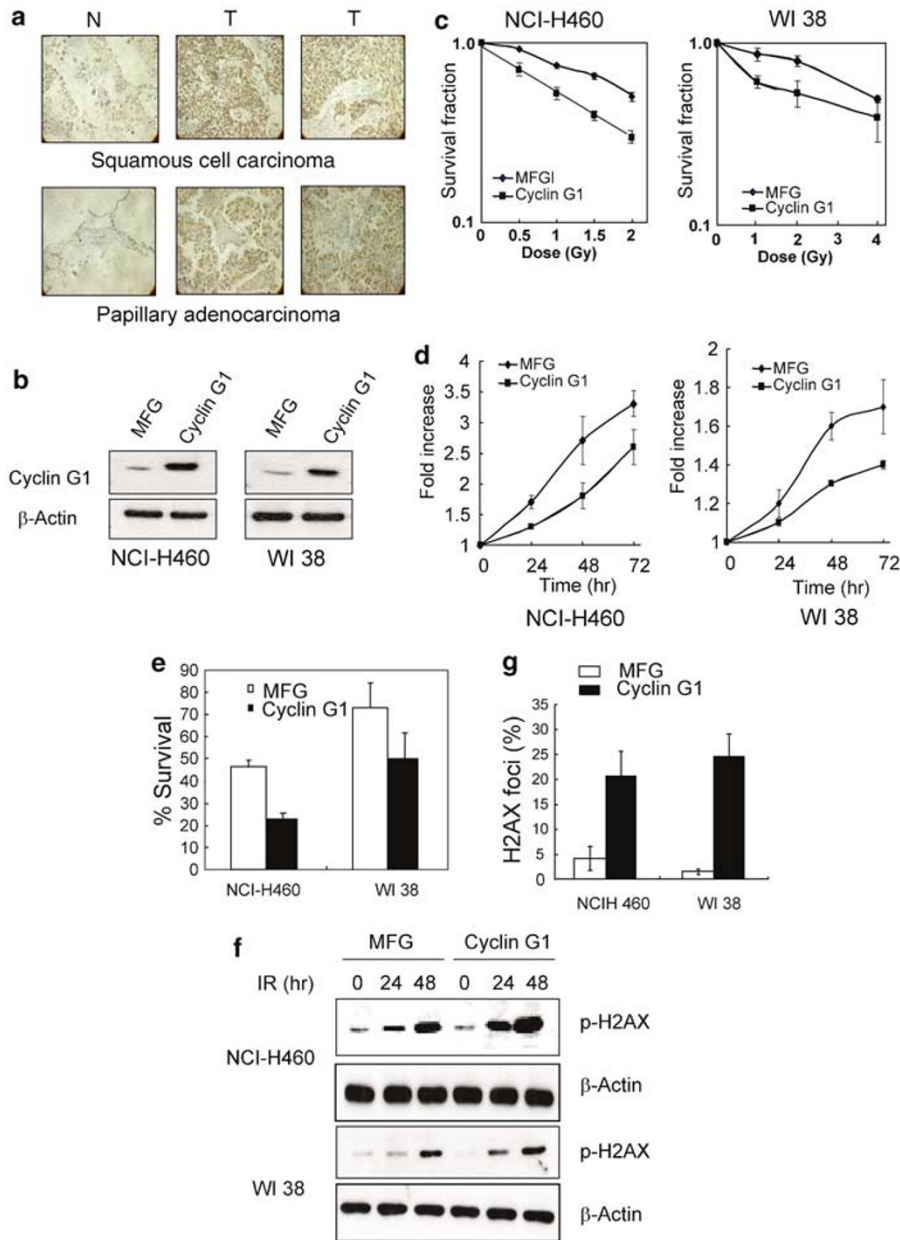


Figure 1 Effects on radiation induced growth inhibition and cell death by cyclin G1. (a) Immunohistochemistry for cyclin G1 was performed using the sections of tissue microarray. Antibody was detected by the aminobenzidine method that produces brown color. N: normal lung tissue; T: lung tumor tissue B. Western blot analysis for cyclin G1 in control and cyclin G1 transfected cells (b). Colony forming assay after various doses of radiation (c), Trypan blue dye exclusion assay after 5 Gy radiation (d) and PI staining at 48 h after 5 Gy radiation (e) were performed in control or cyclin G1 overexpressed cells. Western blot analysis for phospho-H2AX was done at indicated time points of 5 Gy radiation (f) and γ -H2AX foci at 48 h after 5 Gy radiation (g) were examined using fluorescent microscopy. Results are means and standard deviation of three independent experiments

Treatment of siRNA for p53 (sip53) inhibited cyclin B1 expression as well as cyclin G1 expression. However, when cyclin G1 expression vector was additionally transfected to sip53 treated cells, the level of both cyclin G1 and cyclin B1 expression was restored to that of cyclin G1 overexpressing cells, even though p53 expression was still intact (Figure 5b). These results suggest that cyclin B1 regulation by cyclin G1 is independent of p53. To determine whether treatment of siB1 as well as siG1 restored cyclin G1-mediated phenomena, the expression of p21 protein and histone H2AX was examined.

Decreased expression of p21 and increased expression of MPM-2 by cyclin G1 was restored to the level of the control cells by treatment of both siB1 and siG1, suggesting that cyclin G1-mediated phenomena were cyclin B1 dependent (Figure 5c). In fact, when the expression vector of cyclin G1 or cyclin B1 was transiently transfected, the results corroborated our hypothesis (Figure 5d). The expressions of p21 and phospho-CDC2 were inhibited by cyclin B1 overexpression, which corresponded well with the results by cyclin G1 overexpression.

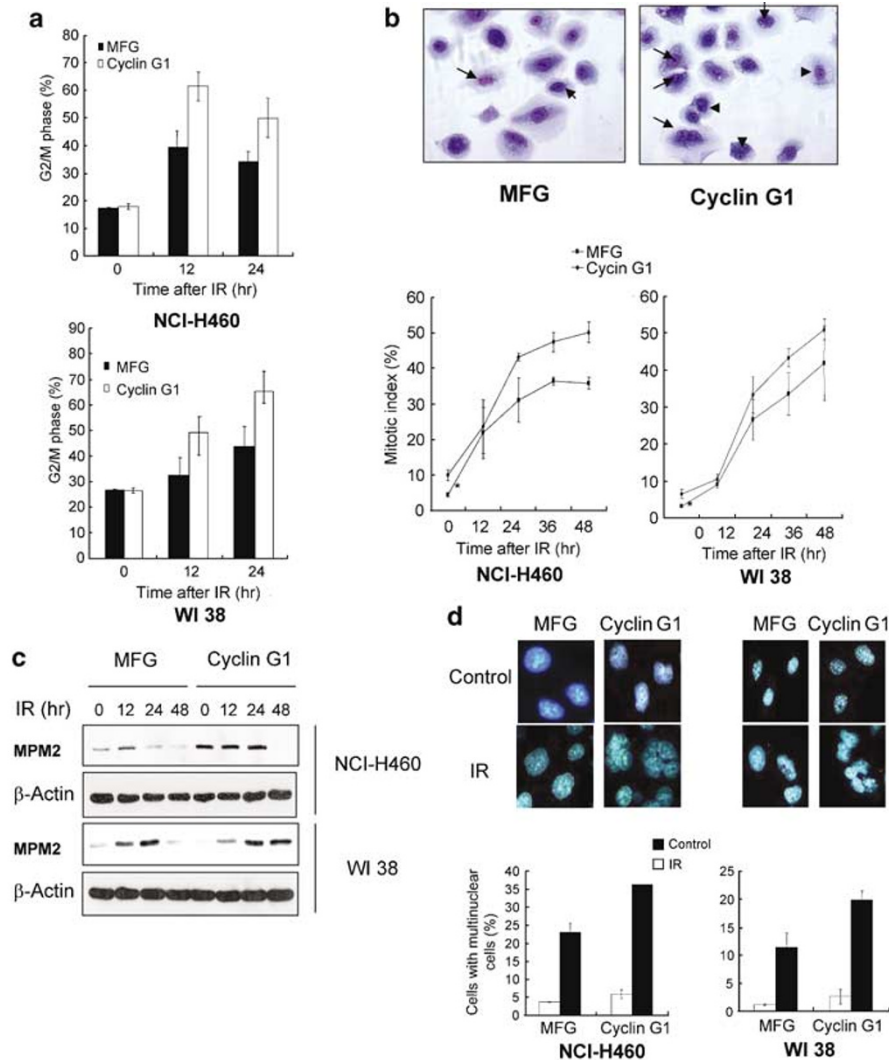


Figure 2 Radiation-induced G2 arrest was abrogated by cyclin G1. (a) At 12 and 24 h after radiation, flow cytometry analysis after PI staining was determined in control and cyclin G1 overexpressing cells. (b) Morphological analysis after Giemsa staining in 5 Gy radiation treated NCI-H460 cells. Arrow indicated mitotic cells (upper) and mitotic index was calculated (lower). (c) Western blot analysis for MPM-2 was performed at indicated time points of 5 Gy radiation. (d) Multinucleated giant cells were counted at 48 h after 5 Gy radiation. Results are means and standard deviation of three independent experiments

Radiation sensitivity by cyclin G1 was abolished by treatment of cyclin B1 siRNA

To elucidate whether increased sensitivity and mitotic arrest by radiation was mediated by cyclin B1, cyclin G1 overexpressing cells were treated with siB1. As seen in Figure 6a and b, increased cell death and G2/M phase by radiation in cyclin G1 overexpressing cells was attenuated by treatment with siB1 as well as siG1. Increased expression of cyclin B1 and H2AX by radiation in cyclin G1 overexpressing cells was also diminished whereas the reduced expression of p21 by cyclin G1 was restored (Figure 6c). Indeed, when mitotic index was examined under a microscopy after Giemsa staining, increased number of radiation induced mitotic cells by cyclin G1 was reduced after siG1 or siB1 (Figure 6d). These results indicate that the increase of mitotic cells and cell death by radiation was mediated by cyclin B1 in cyclin G1 overexpressing cells.

Cyclin G1 transcriptionally regulated cyclin B1 expression

Since siRNA for cyclin G1 reduced the expression level of cyclin B1 protein, we examined whether cyclin G1 regulates cyclin B1 transcriptionally or post-translationally. Real-time RT-PCR analysis revealed that mRNA of cyclin B1 was increased in the cyclin G1 overexpressing cells, while the increased mRNA of cyclin B1 was reduced when siRNA for cyclin G1 was cotransfected (Figure 7a). Increased cyclin B1 promoter activity also indicated that cyclin G1 was directly involved in transcriptional level of cyclin B1 (Figure 7b). Since cyclin G1 has been shown to promote cell growth,⁶ nocodazole was employed to inhibit cell growth in order to exclude the possibility that cyclin B1 regulation by cyclin G1 is not associated with cyclin G1-mediated growth facilitation. Increased cyclin B1 promoter activity was still present in the cyclin G1 overexpressing cells, even though nocodazole was

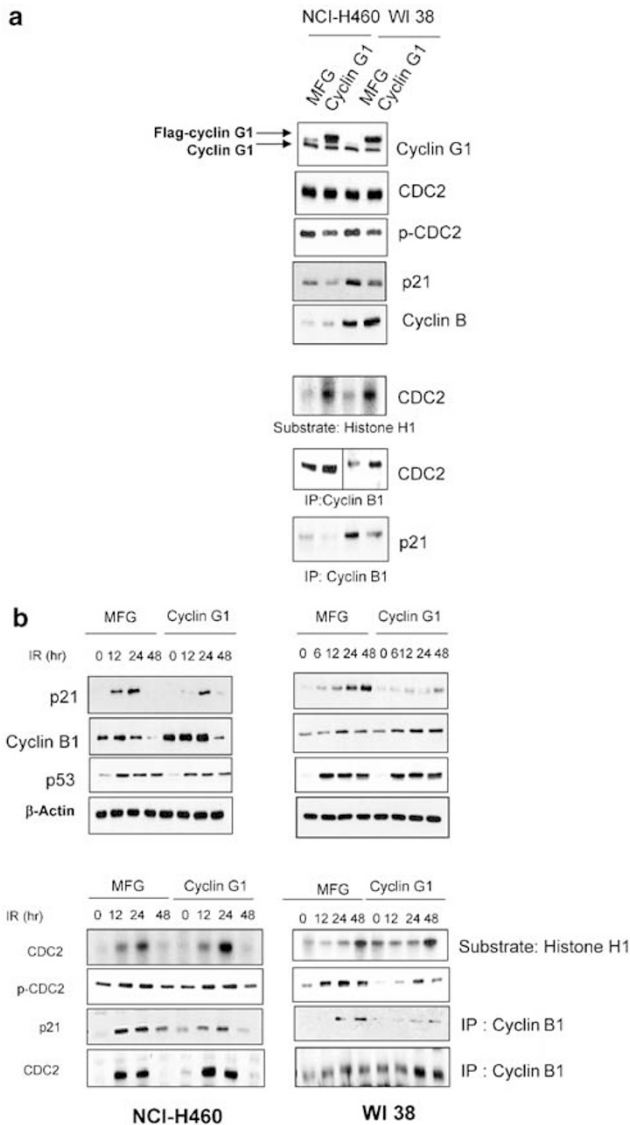


Figure 3 Cyclin G1 inhibited cell cycle regulators related to the radiation-induced G2 arrest. (a) Western blot or immunoblotting after immunoprecipitation was performed in control and cyclin G1 overexpressing cells. (b) At indicated time points of 5 Gy radiation, Western blot or immunoblotting after immunoprecipitation was performed. Result represents one of three independent experiments

used to inhibit cell growth, accompanied with increased cyclin B1 protein levels (Figure 2d).

Discussion

Irradiation elicits various cellular response such as protein synthesis, activation of proteins as well as activation of cell cycle checkpoints that delay the progression of cell growth.²² Irradiation activates checkpoints at the transition from G1 to S phase, in the S phase, and at the transition from the G2 phase to mitosis.²² Or if the damage is too much to handle, cells induce cell death or apoptosis. Here, we noted that understanding of the mechanisms by which cyclin G1 regulates the checkpoints for cell survival or for apoptosis are scarce.

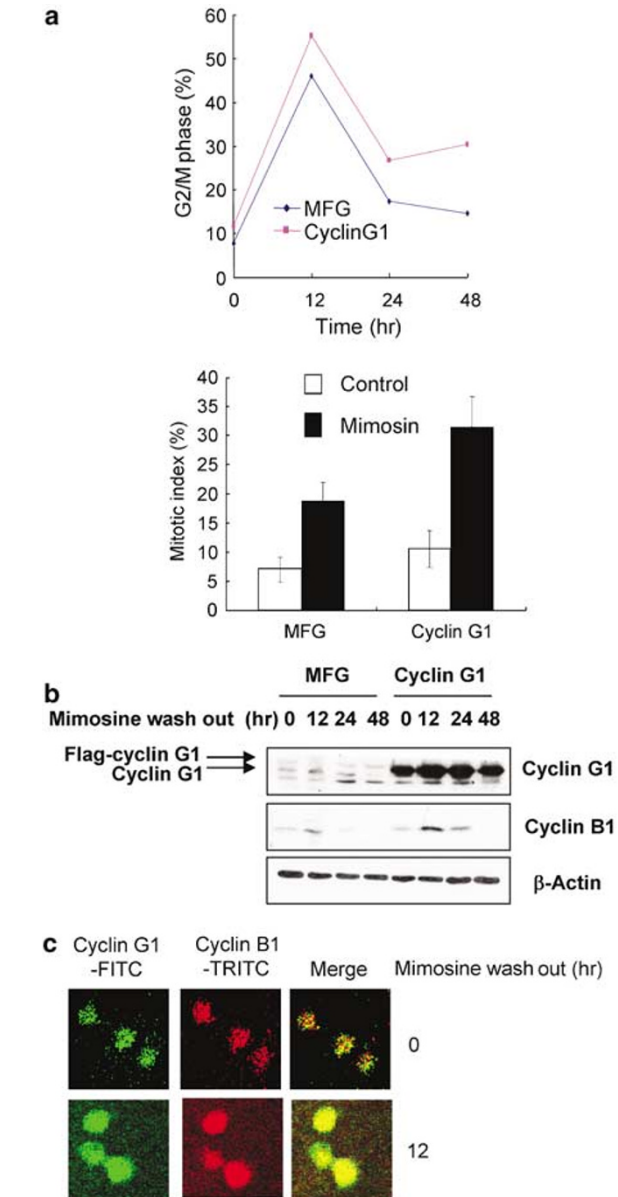


Figure 4 Cyclin G1 predominantly expressed in G2/M phase. (a) After overnight treatment with 200 μ M mimosine, cells were cultured with new media. Flowcytometry at indicated time points of mimosine wash-out was performed (upper) and multinucleated giant cells were counted at 12 h of mimosine wash-out (lower). (b) Western blot analysis was performed at indicated time points of mimosine wash-out (upper) and immunofluorescence analysis using anticyclin G1 antibody for the time indicated. PI was used as counter staining for nucleus (lower). Results are means and standard deviation of three independent experiments

In the present study, we showed that cyclin G1, which is one of the radiation response genes in peripheral blood lymphocytes¹⁷ and frequently overexpressed in tumor tissues,^{5,6,18} was found to inhibit radiation-induced G2 arrest with consequent increase in mitotic cells. Increased mitotic index by cyclin G1 seems to result from transcriptional upregulation of cyclin B1. Hence, our findings demonstrate that cyclin G1 enhanced radiation sensitivity by overriding G2 arrest through upregulation of cyclin B1 and mitotic index.

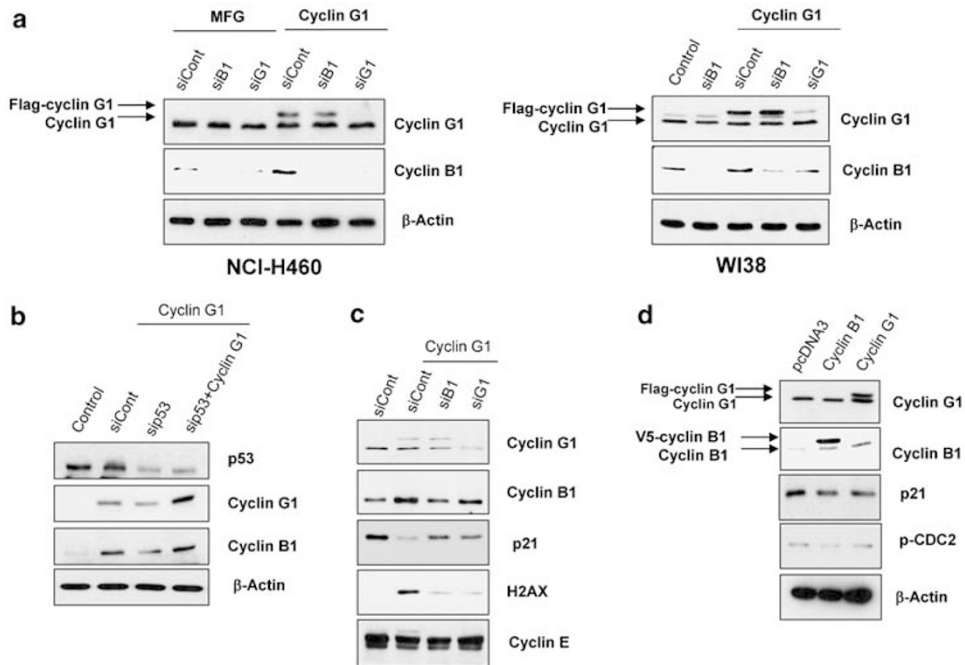


Figure 5 Cyclin G1 regulated cyclin B1 expression. (a, c) Control or cyclin G1, or cyclin B1 siRNAs (siCont, siG1 or siB1) were transfected to control and cyclin G1 overexpressing cells, and Western blot analysis was performed. (b) Control or p53 siRNAs (sip53) with or without additional cyclin G1 transfection were added to control and cyclin G1 overexpressing NCI-H460 cells, and Western blot analysis for cyclin B1 and cyclin G1 was performed. (d) Cyclin G1 or cyclin B1 was transiently overexpressed in NCI-H460 cells, and Western blot was performed. Results present one of three independent experiments

In our tissue array data, cyclin G1 was frequently overexpressed in tumor tissues, including astrocytoma, melanoma, esophagus carcinoma, lung carcinoma, breast carcinoma, cervix, uterus, ovary, prostate and liver cancer (data not shown except for lung carcinoma). A report also indicated similar overexpression of cyclin G1 in leiomyoma tissue.⁶ However, exact mechanism of how cyclin G1 was overexpressed in tumor tissues remains unclear, even though no detectable mutation of p53 was observed. Cyclin G1 expression is specific to nucleus, and a report suggests that cyclin G1 may function by association with replication foci protein such as proliferating cell nuclear antigen.⁶ Ectopic overexpression of cyclin G1 in human RKO colon carcinoma cell line accelerated cell growth, and transfection of normal human fibroblasts with cyclin G1 increased the proliferative lifespan of the fibroblasts,²³ suggesting that cyclin G1 may be associated with oncogenic potential.

Although the precise function of cyclin G1 remains unclear, it has been suggested to contribute to G2/M arrest of cells in response to DNA damage²⁴ and play a role in apoptosis.⁸ Thus, the function of cyclin G1 may involve growth control, induction of apoptosis or DNA repair following cellular damage caused by various stress stimuli.

In the present study, cyclin G1 overexpression in NCI-H460 and WI38 lung cells was shown to increase radiosensitivity and radiation damage when H2AX phosphorylation was detected, suggesting that cyclin G1 sensitized cells to radiation damage. Indeed, transplantation of cyclin G1 overexpressing NCI-H460 cells to nude mice dramatically inhibited growth rate by radiation (data not shown), suggesting that cyclin G1 overexpressing tumors had increased sensitivity to

radiation (Figure 1) and are more prone to radiation therapy. To elucidate the mechanism of radiation sensitization by cyclin G1, cell cycle distribution and morphological analysis were performed after radiation. Inhibition of radiation-induced G2 arrest and increase of mitotic cells by radiation were evident in cyclin G1 overexpressed cells. The CDC2–cyclin B1 complex plays a critical role,²⁵ and the kinase activity of CDC2 is controlled during the cell cycle by both its association with cyclin B1 and phosphorylation status on the inhibitory phosphorylation sites. Cyclin B1 overexpression is also observed in various carcinomas, including esophageal squamous cell carcinoma, laryngeal squamous cell carcinoma and colorectal carcinoma.^{26–29} In the present study, cyclin G1 overexpression increased cyclin B1 expression, accompanied with increased CDC2 kinase activity with or without radiation, whereas the expression of p21, which is known as a broad range CDK inhibitor, was downregulated, suggesting inhibition of radiation-induced G2 arrest by cyclin G1. Because cyclin B1 expression by cyclin G1 was transcriptionally regulated (Figure 7), we suggest that increased cyclin B1 by cyclin G1 may be involved in CDC2 kinase activity with no change of CDC2 protein level, downregulation of p21 protein level, and finally inhibiting G2 arrest. Indeed, when cyclin B1 was overexpressed, the levels of phospho-CDC2 and p21 were inhibited (Figure 5). We do not know the mechanism of how cyclin B1 is regulated by cyclin G1. However, cyclin G1 transcriptionally activated cyclin B1 apparently independent of growth control (Figure 7b).

In mammalian cells, G2–M transition is controlled by the mitotic cyclin B1.³⁰ The expression of cyclin B1 is regulated during the cell cycle, peaking at the G2–M transition. It has

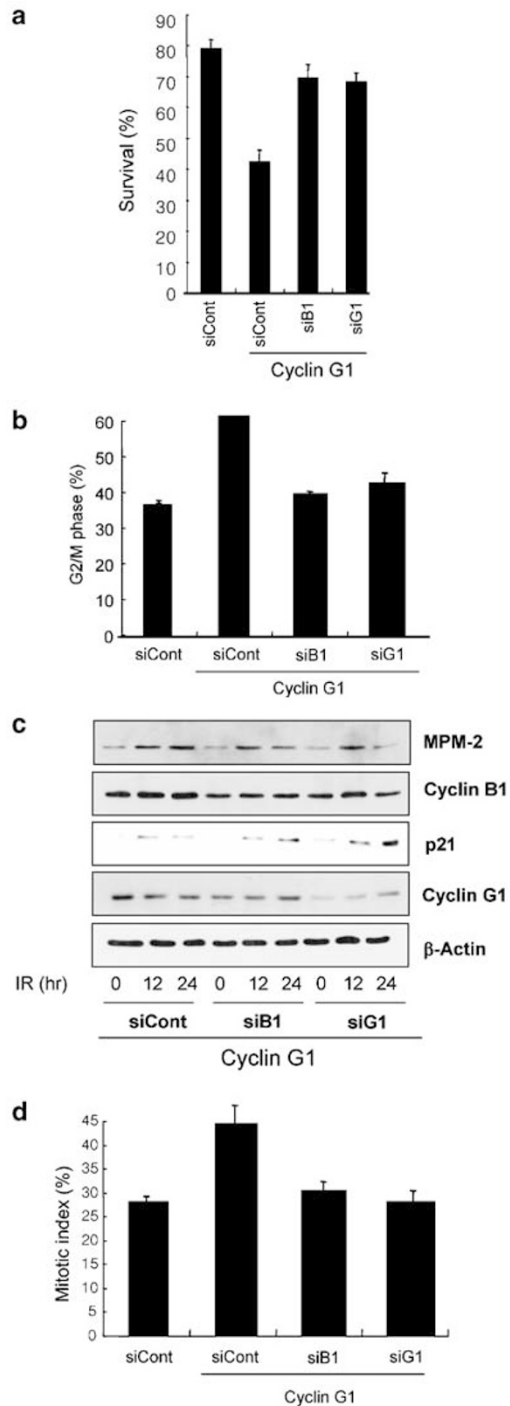


Figure 6 Radiation sensitivity by cyclin G1 was mediated by cyclin B1. Control or cyclin G1, or cyclin B1 siRNAs (siCont, siG1 or siB1) were transfected to control and cyclin G1 overexpressing NCI-H460 cells. Flow cytometric analysis after PI staining was performed (a), cell cycle distribution after PI staining was determined (b), and mitotic arrested cells were counted (d). (c) At indicated time points of 5 Gy radiation, Western blot was performed. Results are means and standard deviation of three independent experiments

been known that the transcriptional activity of human cyclin B1 promoter is induced during the G2–M transition and repressed in G1.^{31–34} However, it has not yet been demonstrated whether the cyclin B1 promoter retains transcriptional activity

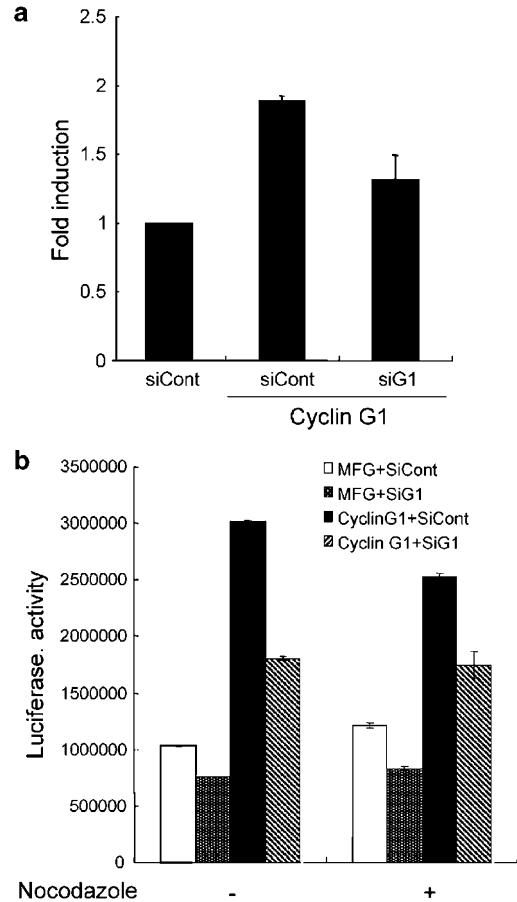


Figure 7 Cyclin G1 regulated cyclin B1 transcriptionally. Real-time RT-PCR analysis (a) and luciferase reporter gene assay for cyclin B1 promoter (b) with or without 12 h nocodazole (1 μ M) treatment were performed at 24 h of transient transfection with siRNAs of control or cyclin G1 (siCont or siG1) in control and cyclin G1 overexpressing cells. Results are means and standard deviation of three independent experiments.

during mitosis. After DNA damage by ionizing radiation, mammalian cells are arrested at the transition from G1 to S phase or from G2 to M phase in the cell cycle, thus preventing DNA replication and mitosis in the presence of DNA damage. A recent report that the expression of wild-type p53 in human cells leads to an increase of G2 as well as G1 populations of the growth-arrested cells³⁵ suggests that p53 regulates the G2–M transition. However, in a certain system, p53 expression does not clearly indicate the regulatory role of p53, specifically in G2–M transition. In our present experiment, p53 expression was not changed by cyclin G1 overexpression, and p53 induction patterns by radiation were not different between the control and cyclin G1 overexpressing cells, either. Treatment of p53 siRNA reduced cyclin G1 and cyclin B1 expressions. However, when the cyclin G1 expression which is one of p53 target genes was recovered, p53 protein expression was still downregulated, while cyclin B1 expression was inhibited (Figure 5), suggesting that cyclin B1 regulation by cyclin G1 is independent of p53 expression. Moreover, radiosensitivity by cyclin G1 was correlated with cyclin B1. Treatment of cyclin B1 siRNA restored clonogenic survival after radiation to the control level, and similar

tendency was also observed with the treatment of cyclin G1 siRNA. The number of cells with G2/M phase and mitotic cells by radiation were also reduced to the level of control cells by cyclin B1 siRNA treatment, while cyclin G1 overexpressed cells showed high mitotic cells, suggesting that cyclin B1 expression by cyclin G1 is responsible for the inhibition of G2 arrest by radiation, subsequently resulting in increased cell death. One defining feature of cells undergoing this form of cell death is the presence of giant and multinucleated cells (Figure 2d). Some reports indicate that escape of damaged cells from G2 arrest shares a common pathway with apoptosis-inducing cell death^{36–38} and our results also showed that cyclin G1 potentiated cell death via inhibition of radiation-induced G2 arrest following ionizing radiation.

In conclusion, the above results indicate that cyclin G1 overcame radiation-induced G2 arrest and increased cell death through transcriptional activation of cyclin B1, and that cyclin G1 overexpressing cancer tissue might clinically be more sensitive to radiation.

Materials and Methods

Reagents

Mimosine, nocodazole and monoclonal antibody for phospho-CDC2 were purchased from Sigma (St. Louis, MO, USA), monoclonal antibodies for cyclin G1, cyclin B1, CDC2, p21, and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and phospho-H2AX, MPM-2 and histone H3 were from Upstate (Charlottesville, VA, USA).

Plasmids and transfection

For overexpression of cyclin G1, human cyclin G1 cDNA was cloned into murine leukemia virus MFG vector. This vector was constructed by an insertion of FLAG-tagged cyclin G1 cDNA between *NcoI* restriction site and *BamHI* restriction site on *env* region of wild-type virus genome. The MFG-cyclin G1 vector was transfected into the BD RetroPack™ PT67 cell line (Cat. No. 631510, BD Biosciences Clontech, Becton Drive, Franklin Lakes, NJ, USA). Virus produced by these cells was used to overexpress FLAG-cyclin G1 in NCI-H460 or WI38. For overexpression of cyclin B1, human cyclin B1 cDNA was cloned into pcDNA4/V5-His (Invitrogen). This vector was constructed by an insertion of cyclin B1 cDNA between *BamHI* restriction site and *XhoI* restriction site on pcDNA4/V5-His, which contains C-terminal V5-epitope. The cyclin B1 promoter-firefly luciferase reporter construct hB1-Luci was kindly provided by Kurt Engeland.³⁹ Predesigned siRNA for human cyclin G1, cyclin B1, p53 (Ambion Cat# 19706, 51187, 16706) and negative control siRNA were purchased from Ambion, Inc. (Austin, TX, USA). The cells were transfected with the siRNAs for 48 h with the use of Lipofectamin™ 2000 (Invitrogen). A mixture of 250 μ l of Opti-MEM medium (Invitrogen) and 20 μ l of Lipofectamin™ 2000 was incubated for 5 min at room temperature and was then combined with 100 nM siRNA diluted with 250 μ l of Opti-MEM. The resulting mixture (500 μ l) was incubated for 20 min at room temperature to allow complex formation and then overlaid onto each well, containing the cells, in a final 2.5 ml volume per 60-mm dish.

Cell culture

NCI-H460 human non-small-cell carcinoma and WI38 human lung fibroblast cells were cultured in RPMI (GIBCO, Gaithersburg, MD,

USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS, GIBCO) and antibiotics at 37°C in a humidified incubator with 5% CO₂.

Irradiation

Cells were plated in 60 mm dishes and incubated at 37°C under humidified 5% CO₂ in culture medium until 70–80% confluent. Cells were then exposed to γ -rays with ¹³⁷Cs γ -ray source (Atomic Energy of Canada, Ltd, Canada) with dose rate of 3.81 Gy/min.

Colony-forming assay

Clonogenicity was examined by colony-forming assay, as described previously.^{40,41} Cells were seeded into 60 mm dishes at densities to produce ~500 colonies per dish in the control and were incubated for 7–14 days. Colonies were fixed with a mixture of 75% methanol and 25% acetic acid, and stained with 0.4% Trypan blue. The number of colonies consisting of 50 or more was scored.

Detection of cell death

Cells were plated on glass slides and irradiated. After 24 or 48 h, cells were fixed in 70% ethanol, washed with PBS, and incubated with 1 μ g/ml bisbenzimidazole trihydrochloride in PBS (Hoechst 33258) for 30 min in dark. Specimens were viewed by fluorescence microscopy using Olympus BX-40 microscope. At least 200 cells for each determination were scored. Apoptosis was characterized by chromatin condensation and fragmentation

Cell cycle analysis

For cell cycle analysis, cells were fixed in 80% ethanol at 4°C for at least 18 h. The fixed cells were then washed once with PBS-EDTA and resuspended in 1 ml of PBS. After the addition of 10 μ l each of propidium iodide (5 mg/ml) and RNase (10 mg/ml), the samples were incubated for 30 min at 37°C and analyzed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Immunoprecipitation

Cells (1×10^7) were lysed in immunoprecipitation buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40). After centrifugation (10 min at 15 000 g), to remove particulate material, the supernatant was incubated with antibodies (1 : 100) against anti-p21 p27 or cyclin B1 with constant agitation at 4°C. The immunocomplexes were precipitated with protein A-Sepharose (Sigma) and analyzed by SDS-polyacrylamide gel electrophoresis using enhanced chemiluminescence detection (Amersham International).

CDC2 kinase assay

Cell lysates were incubated with CDC2 antibody, and immunocomplexes were collected on protein A-Sepharose beads and resuspended in kinase assay mixture containing [γ -³²P]ATP (NEN Life Science, Boston, MA, USA) and histone H1 (Life Technologies, Inc., Paisley, Scotland, UK) as substrates. Proteins were separated on SDS-polyacrylamide gels, and bands were detected by autoradiography.

Polyacrylamide gel electrophoresis and Western blot

For polyacrylamide gel electrophoresis (PAGE) and Western blot, cells were solubilized with lysis buffer (120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% NP40), the samples were boiled for 5 min, and equal amount of protein (40 μ g/well) was analyzed on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting. Blots were further incubated with horseradish peroxidase-conjugated secondary antibody diluted at 1:5,000, and specific bands were visualized by chemiluminescence (ECL, Amersham International). Autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co.).

Quantitative real-time PCR

Real-time PCR analysis was performed using a DNA Engine2.OPTICON (MJ Reserch) and the LightCycler-FastStart DNA Master SYBR Green I mix (Roche). 3'-locked nucleic acid (LNA) primers were synthesized by Prologo. Reactions were performed in a final volume of 15 μ l, adjusted to 4 mM MgCl₂ and containing 500 nM each of primers and 2 μ l of DNA template. The real-time PCR cycling conditions were as follows: 94°C for 5 min, followed by 32 cycles for 1 min at 94°C, 1 min at 52°C and 2 min at 72°C followed by fluorescence measurement. The polymerization temperature was set to 68°C to allow accurate fluorescence measurements, because of the low-melting temperature of the PCR products analyzed. Following PCR, a thermal melt profile was performed for amplicon identification. To determine the Ct, the threshold level of fluorescence was set manually in the early phase of the PCR amplification.

Immunofluorescence analysis

For immunofluorescence analysis, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and then washed three times with PBS. Cells were then incubated with anticyclin G1 diluted 1:200 in PBS with 5% FBS for 1 h at room temperature in a humidified chamber. Excess antibody was removed by washing coverslips three times with PBS. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Dako, Produktionsvej, Denmark), at 1:200 dilution in PBS with 5% FBS for 4 h, and then incubated with 0.5 μ g/ μ l propidium iodide (Molecular Probes, Inc., Eugene, OR, USA) for 5 min at room temperature. After washing three times with PBS, coverslips were mounted onto microscope slides using ProLong antifade mounting reagent (Molecular Probes). The slides were analyzed by a confocal laser-scanning microscope (Leica Microsystems).

Immunohistochemistry

Various cancer tissues with corresponding normal tissues were arrayed from formalin-fixed and paraffin-embedded tissues of AccuMax array (Petagen Inc., Seoul, Korea), and these arrayed slides were used for the immunostaining of cyclin G1. Deparaffinization and rehydration were performed using xylene and alcohol, and pretreated slides were incubated in 3% H₂O₂ for 13 min to remove endogenous peroxidase activity. The tissue was reacted with primary anticyclin G1 for 2 h in a humid chamber at room temperature and washed with PBS for 10 min, and the sections were incubated for 20 min at room temperature with secondary antibody. After additional incubation with streptavidin-HRP for 10 min, chromogen reaction was developed for 10 min in 3,3'-diaminobenzidien. The sections

were counterstained with Harris' hematoxylin, dehydrated, and mounted with coverslips.

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