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Inverse Dose-rate-effects on the Expressions of Extra-cellular Matrix-related Genes in Low-dose-rate γ-ray Irradiated Murine Cells

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Low-dose-rate radiation/p53/Extra-cellular matrix/Gene expression.

Based on the results of previous microarray analyses of murine NIH3T3/PG13Luc cells irradiated with continuous low-dose-rate (LDR) γ-ray or end-high-dose-rate-irradiations (end-HDR) at the end of the LDR-irradiation period, the inverse dose-rate-effects on gene expression levels were observed. To compare differences of the effects between LDR-irradiation and HDR-irradiation, HDR-irradiations at 2 different times, one (ini-HDR) at the same time at the start of LDR-irradiation and the other (end-HDR), were performed. The up-regulated genes were classified into two types, in which one was up-regulated in LDR-, ini-HDR-, and end-HDR irradiation such as *Cdkn1a* and *Ccng1*, which were reported as p53-dependent genes, and the other was up-regulated in LDR- and ini-HDR irradiations such as *pro-collagen TypeIa2/Col1a2*, *TenascinC/Tnc*, and *Fibulin5/Fbln5*, which were reported as extra-cellular matrix-related (ECM) genes. The time dependent gene expression patterns in LDR-irradiation were also classified into two types, in which one was an early response such as in *Cdkn1a* and *Ccng1* and the other was a delayed response such as the ECM genes which have no linearity to total dose. The protein expression pattern of Cdkn1a increased dose dependently in LDR- and end-HDR-irradiations, but those of p53Ser15/18 and MDM2 in LDR-irradiations were different from end-HDR-irradiations. Furthermore, the gene expression levels of the ECM genes in embryonic fibroblasts from *p53*-deficient mice were not increased by LDR- and end-HDR-irradiation, so the delayed expressions of the ECM genes seem to be regulated by p53. Consequently, the inverse dose-rate-effects on the expression levels of the ECM genes in LDR- and end-HDR-irradiations may be explained from different time responses by p53 status.

INTRODUCTION

Several transcription factors are activated in a variety of cell lines by low-dose (LD) γ-rays or X-rays, and the regulation mechanisms of p53 transcription in stress responses to high-dose (HD) and high-dose-rate (HDR)-irradiation have been well studied. Low-dose-rate (LDR)-irradiation at 20–500 mGy activated several p53-dependent stress genes such as *p21/Cdkn1a* and *Gadd45* in ML-1 cells.[1)] LDR-irradiation before HDR-irradiation suppressed the accumulation of p53 and Bax proteins leading to apoptosis.[2)] DNA binding of transcription factors such as Oct, NF-κB, HNF, NF-AT, and the KLF family were also identified in LD X-ray-irradiated cells by a competition assay.[3)] LD (100 mGy)-irradiation activates NF-κB transcription factor transiently with maximal induction at 500 mGy.[4)] DNA-binding activity of NF-κB was also observed in X-ray-irradiated endothelial cells with a maximum level at 500 mGy.[5)]

Changes in protein synthesis levels after irradiation with LD-γ-ray or LD-X-ray were mostly associated with signal transduction pathways. For example, LD irradiation induced an increase of TGF-β1, a cytokine closely associated with radiation-induced fibrosis.[6–9)] LD-irradiation (100 mGy) was shown to activate TGF-β1 in mammary epithelial cells.[7)] In another study, allogeneic hepatoma cells implanted into rat irradiated at a daily dose of 200 mGy for 14 days induced TGF-β1 production.[8)] IL-1-stimulated endothelial cells also showed TGF-β1 production after LD-X-ray irradiation between 300 and 700 mGy.[9)] Furthermore, ERK1/2 and MEK kinases also enhanced cell proliferation and differentiation after LD-X-ray irradiation.[10)] These findings suggest

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that changes in signal transduction after LD-X-rays or LD-γ-rays have important roles in variable biological responses and phenomena.

However, there have been few studies on the relationship between gene expression, total dose, and dose-rate in LDR-irradiation. We previously reported the enhanced expressions levels of *Cdkn1a*, *Ccng1* in murine NIH/PG13Luc cells following continuous LDR γ-ray -irradiation at a dose-rate of 1 to 100 mGy/hr.[11] In addition, three extra-cellular matrix-related genes (*Col1a2*, *Tnc*, *Fbln5*), were identified as highly expressed in only LDR-irradiated murine NIH3T3/PG13Luc cells from a previous microarray analysis.[11] These five genes, *Cdkn1a*, *Ccng1*, *Col1a2*, *Tnc*, and *Fbln5* were selected for the present analysis to compare gene expressions levels according to irradiation time (total dose) between LDR- and HDR-irradiation.

MATERIALS AND METHODS

Cell Culture

C3H/*p53*/knockout-mouse embryonic fibroblast (C3H/*p53*/KO-MEF) cells and C3H/p53/wild type-mouse embryonic fibroblast (C3H/*p53*/WT-MEF) cells were established from embryos that were born from a Riken C3H/HeN-TgH (*p53*) mouse (Acc. No.CDB 0001K).[12] Murine immortalized NIH3T3/PG13Luc cells[11,13] with a stably integrated p53-responsive reporter plasmid were incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), at 37°C in a 5% CO_2 atmosphere before and after irradiation. C3H/*p53*/KO-MEF and C3H/*p53*/WT-MEF cells were incubated in Advanced Dulbecco's Modified Eagle's Medium (ADMEM) supplemented with 10% fetal bovine serum (FBS), at 37°C in a 5% CO_2 atmosphere before and after irradiation. These studies using cultured murine cells were approved by the Ethical Procedures Committee of our institution.

^{137}Cs γ-ray irradiation for real-time PCR analysis

A Gamma-Simulator (Fuji Electronic Systems Company, Japan) was used for irradiation with LDR ^{137}Cs γ-ray at dose rates of 1, 15, 60, and 90 mGy/hr for 72 hr (total doses of 72, 1080, 4320, and 6480 mGy, respectively). A Gamma-Cell (MDS Nordion, Canada) was used for HDR-irradiations (0.9 Gy/min) with ^{137}Cs γ-ray (total doses of 72, 1080, 4320, and 6480 mGy, respectively). Total doses of ^{137}Cs γ-ray irradiation were measured with a photo-luminescent dosimeter

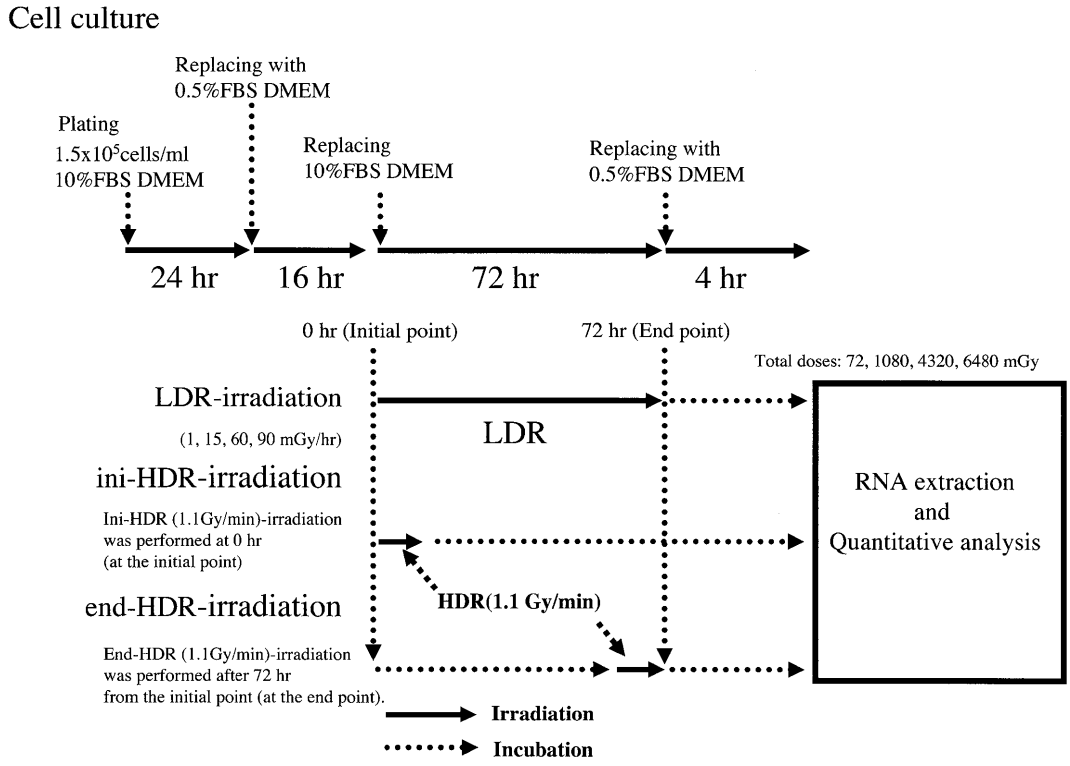


Fig. 1. Scheme for cell cultures and γ-ray irradiation of NIH/PG13Luc cells: Cells were irradiated for 72 hr with LDR γ-rays. For ini-HDR-irradiation, cells were irradiated for several minutes with HDR γ-rays at the time corresponding to the initial point (0 hr) of LDR-irradiation and cultured for 72 hr under non-irradiated conditions. For end-HDR-irradiation, cells were cultured for 72 hr under non-irradiated conditions, and then irradiated for several minutes with HDR γ-rays at the time corresponding to the end-point (72 hr) of LDR-irradiation. RNA extraction and quantitative analysis of gene expression were performed at 4 hr after 72 hr culture.

(Asahi Technoglass Corporation, Funabashi, Japan).

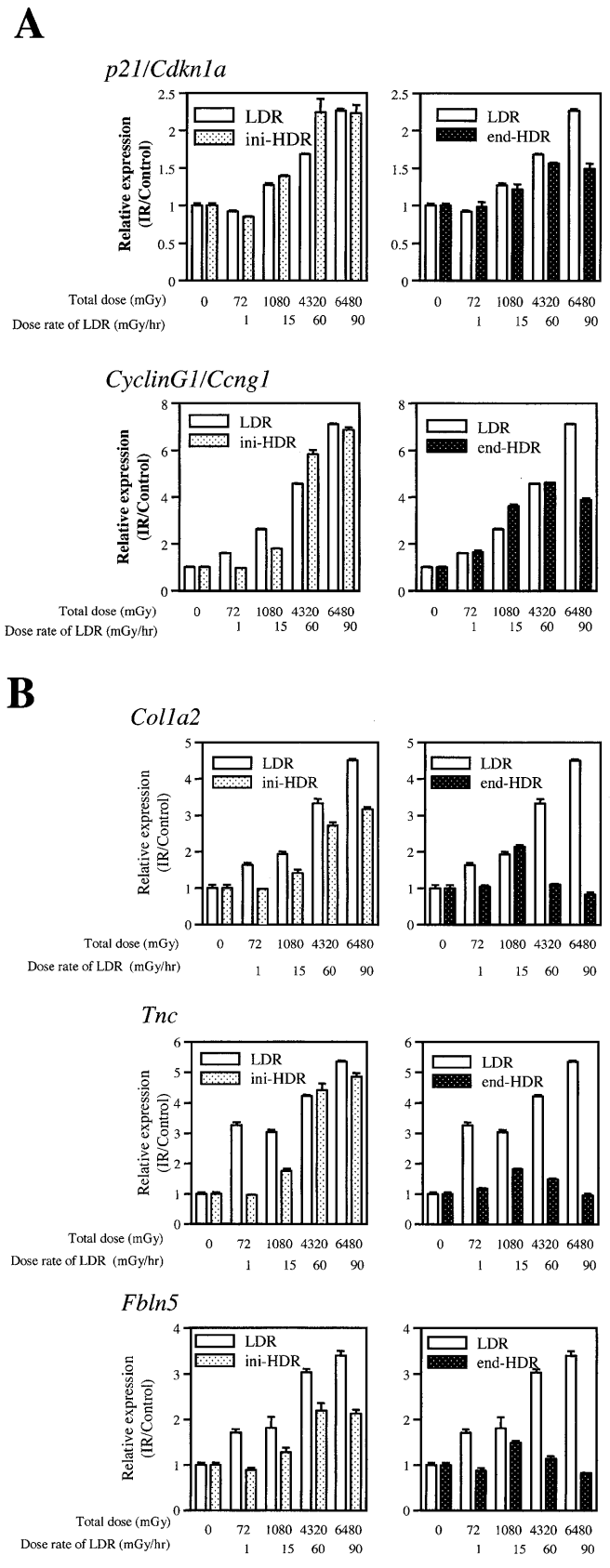
Schedule for cell cultures and γ-ray irradiation

As LDR-irradiation needs a longer irradiation period than HDR-irradiation for the same total dose, it is necessary to compare changes in genes expression levels among differences in irradiation time. HDR-irradiation was performed at either the initial point of 0 hr (ini-HDR) or the end point of

Table 1. List of up-regulated genes in low-dose-rate (100 mGy/h) irradiation from previous microarray analysis

GenBank	Name	ERs (LDR/HDR > 2.0)
AA066816	inhibitor of DNA binding 3 (Id3)	6.07
AA437518	**fibulin 5**	**3.76**
AA273494	vitamin D receptor	3.59
AA822067	epoxide hydrolase 1, microsomal (Ephx1)	3.22
W61385	protein-tyrosine sulfotransferase 1	3.21
AA880094	ESTs	3.01
W18822	growth arrest and DNA-damage-inducible 45 gamma	3.00
W53787	procollagen, type IV, alpha 2	2.99
AA267178	cytochrome P450, 1b1, benz[a]anthracene inducible	2.96
AA177698	programmed cell death 4	2.94
AA071995	semaphorin cytoplasmic domain-associated protein 3A	2.91
W12942	**tenascin C**	**2.89**
AA210242	mage-d2 protein	2.88
AA050300	cold inducible RNA-binding protein	2.88
AI425767	**procollagen, type I, alpha 1**	**2.86**
AA798297	procollagen, type I, alpha 2	2.77
AA106834	ESTs	2.77
AI587823	ESTs, highly similar to inwardly rectifying potassium channel Kir5.1 [M.musculus]	2.75
AA792297	procollagen, type V, alpha 1	2.75
AA718688	disintegrin and metalloprotease domain 23	2.55
AA770854	procollagen C-proteinase enhancer protein	2.48
AI536344	ESTs	2.40
AA726040	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1	2.35
AI385983	RIKEN cDNA 4933439C20 gene	2.35
AA880550	aminolevulinic acid synthase 2, erythroid	2.32
AA647231	low density lipoprotein receptor-related protein 6	2.32
AA162153	colony stimulating factor 1 (macrophage)	2.30
AA388038	fibulin 2	2.27
AI020539	secretory leukocyte protease inhibitor	2.18
AA066634	lactate dehydrogenase 2, B chain	2.13
AI391125	adenylate kinase 1	2.12
AA561779	Mus musculus putative nucleotide pyrophosphatase/phosphodiesterase	2.07
AA037995	microfibrillar associated protein 5	2.02
AA596779	B-cell translocation gene 2, anti-proliferative	2.02

ERs (LDR / HDR) show the dose-rate effect for differential expression levels when cells are exposed to the same total dose (7.2 Gy) with both LDR (10 cGy/h) or HDR (0.5 Gy/min). Bold letters indicate selected genes for Real-Time PCR analyses.



72 hr (end-HDR), when LDR-^{137}Cs γ-ray irradiation was completed. Gene expression levels were measured by real-time PCR. Each gene expression level for two different time laps for HDR-irradiation was compared with the expression in LDR-irradiation at the same total dose, as illustrated in Fig. 1. Before irradiation, NIH3T3/PG13Luc cells were pre-incubated in DMEM supplemented with 10% FBS for 24 hr, and then starved in DMEM supplemented with 0.5% FBS for 16 hr to synchronize the cell cycle to obtain accurate and reproducible results in each experiment. Most cells were arrested at G1 phase by starvation (data not shown). The starved cells were incubated in DMEM supplemented with 10% FBS for 72 hr LDR-irradiation, as described above. After irradiation, the cells were cultured in the same conditions as described previously.[11] HDR-irradiation treatments were performed at either the initial (0 hr) or end point (72 hr) of the LDR irradiation.

Re-calculation of microarray results

A previous microarray study was performed using irradiated cells with LDR- or end-HDR-irradiation, similar to present study. The expression ratios for evaluating RNA expression levels were obtained by dividing the value of LDR-^{60}Co γ-ray (100 mGy/hr) irradiation by the value of end-HDR-irradiation using (500 mGy/min) soft X-rays at the same total dose of 7200 mGy, based on previous microarray data.[11] The ratios (LDR/end-HDR) indicate the dose-rate effects on genes expression levels. The cut-off values of the LDR/end-HDR ratios were set as 2 or 0.5 to determine significantly higher or lower gene expression levels.

Real-time PCR

The expression levels of genes detected by microarray were analyzed by real-time PCR (ABI7700; Applied Biosystems, Foster, CA, USA) to quantify the amounts of mRNA expression in NIH3T3/PG13Luc, C3H/*p53*/KO-MEF, and C3H/*p53*/WT-MEF cells. Total RNA isolation and RT-mix preparation were performed as described previously.[11] The cDNAs of *glyceraldehyde-3-phosphate-dehydrogenase/Gapdh* as a control for RNA expressions, *p21/Cdkn1a*, *CyclinG1/Ccng1*, *pro-collagenTypeIa2/Col1a2*, *TenascinC/Tnc*, *Fibulin5/*

Fig. 2. Expression levels of *p21/Cdkn1a* and *CyclinG1/Ccng1* (shown in Fig. 2A), *Col1a2* and *Tnc*, and *Fbln5* (shown in Fig. 2B) in NIH/PG13Luc cells following LDR (1 mGy/hr, 15 mGy/hr, 60 mGy/hr, and 90 mGy/hr) γ-ray irradiation for 72 hr, and HDR (1.1 Gy/min) γ-ray irradiation were analyzed. The same total doses, 72 mGy, 1080 mGy, 4320 mGy and 6480 mGy, were used for both LDR and HDR-irradiation treatments. The relative ratio of mRNA expression levels was calculated as the amount of gene expression following irradiation treatment divided by that of a non-irradiation control (IR/Control).

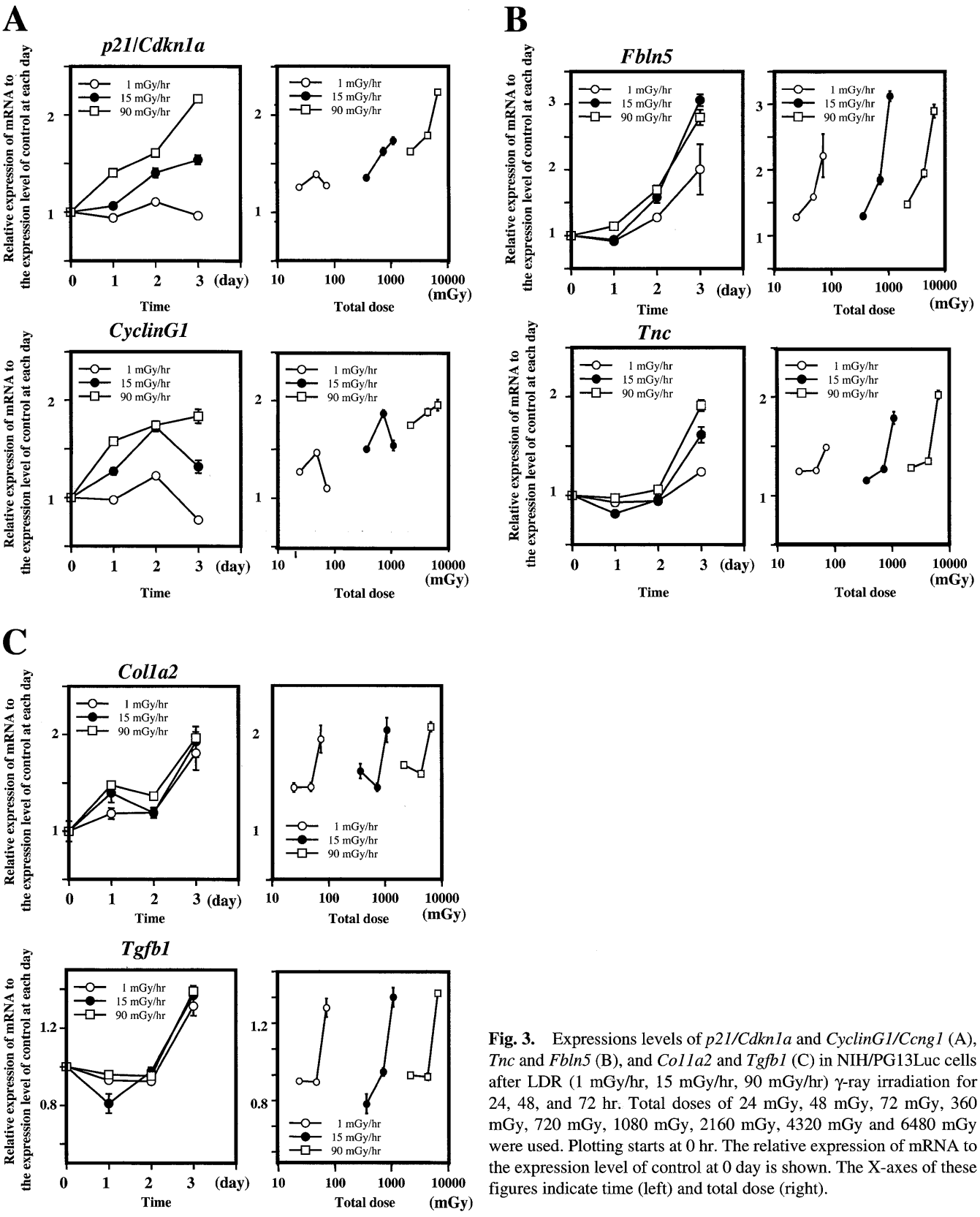


Fig. 3. Expressions levels of *p21/Cdkn1a* and *CyclinG1/Ccng1* (A), *Tnc* and *Fbln5* (B), and *Col1a2* and *Tgfb1* (C) in NIH/PG13Luc cells after LDR (1 mGy/hr, 15 mGy/hr, 90 mGy/hr) γ-ray irradiation for 24, 48, and 72 hr. Total doses of 24 mGy, 48 mGy, 72 mGy, 360 mGy, 720 mGy, 1080 mGy, 2160 mGy, 4320 mGy and 6480 mGy were used. Plotting starts at 0 hr. The relative expression of mRNA to the expression level of control at 0 day is shown. The X-axes of these figures indicate time (left) and total dose (right).

Fbln5, and *TGF-β1/Tgfb1* were amplified and quantified. Primers for sense and anti-sense oligos and TaqMan probes for the detection of *GAPDH*, *p21/Cdkn1a* and *Cyclin G1/Ccng1* were used as described previously.[11] TaqMan probes and primer mixtures for *Col1a2* (Mm00483888_m1), *Tnc* (Mm00495662_m1), *Fbln5* (Mm00488601_m1), and *Tgfb1* (Mm00441724_m1) were used for TaqMan®Gene Expression Assays (Applied Biosystems, Foster, CA, USA).

Western blotting

The expression levels of proteins (p21/Cdkn1a, MDM2, p53ser15/18 and glyceraldehyde-3-phosphate-dehydrogenase/Gapdh) were analyzed by western blotting. Proteins were dissolved in SDS-sample buffer, and were applied for western blotting analysis. These proteins were detected by anti-p21 antibody (Santa Cruz), anti-MDM2 antibody (Santa Cruz), anti-p53ser15 antibody (Cell Signaling), and anti-GAPDH antibody (Santa Cruz), respectively.

RESULTS

Gene expression profiles by microarray

In the previous microarray analysis, numerous genes expressing 2-fold or more increases following LDR-irradiation were detected when their expression levels were compared to those after end-HDR-irradiation.[11] Most of the genes up-regulated by end-HDR-irradiation belonged to genes whose gene expression levels are transcribed by p53.[11] From these findings, genes with LDR/end-HDR ratios of more than 2.0 (LDR/end-HDR > 2.0) by microarray analysis were considered to show higher gene expression by the LDR-irradiation treatment (100 mGy/hr) and to have inverse dose-rate-effects on gene expression, as listed in Table 1. The 34 genes identified included extra-cellular matrix-related (ECM) genes such as *Col1a2*, *Tnc*, and *Fbln5*, while the other genes were related to the cell cycle and metabolic enzymes. On the other hand, genes showing LDR/end-HDR ratios of less than 0.5 (LDR/end-HDR < 0.5) were considered to show lower gene expressions levels following LDR (100 mGy/hr) irradiation (data not shown). The 26 genes identified included cell-cycle-related genes such as *Rbl1* (RB like1/p107), *Mapk9* (JunKinase2/JNK2), *Cdc2a* (cdc2), and *Mcm6* (MCM6). While no ECM genes were included, *Mapk9* and *Cdc2a* were found to be down-regulated by LDR-irradiation. These down-regulated genes were not focused on in the present study.

Expression analysis of Cdkn1a and Ccng1

Previous microarray analysis also showed that *Cdkn1a* and *Ccng1* were up-regulated after both LDR and end-HDR-irradiations treatments,[11] so these two genes were also selected for the present analysis. HDR-irradiation was performed at one of 2 phases, at the initial point (ini-HDR) or end point (end-HDR) of LDR irradiation (Fig. 1). The expression levels of genes after LDR-irradiation were compared to those of each HDR-treatment at different irradiation times. These genes were up-regulated after LDR-, ini-HDR-, and end-HDR-irradiation treatments in proportion to the total doses and dose-rate (Fig. 2A). The end-HDR-irradiation resulted in an immediate response of gene expression, while ini-HDR-irradiation resulted in a delayed response of gene expression.

Expression of extra-cellular matrix-related genes

Since the LDR/end-HDR ratios of ECM genes, such as *Col1a2*, *Tnc* and *Fbln5*, were higher than 2.0 in the microarray analysis (Table 1), the expression levels of such genes were periodically analyzed by real-time PCR in the present study. The gene expression levels of *Col1a2*, *Tnc*, and *Fbln5* were not up-regulated by end-HDR-irradiation (Fig. 2B). On the other hand, these genes were up-regulated by ini-HDR-irradiation at more than 1080 mGy (Fig. 2B). These results indicate that *Col1a2*, *Tnc*, and *Fbln5* genes were up-regulated as a delayed response after ini-HDR-irradiation, but not as an immediate response after end-HDR-irradiation. When end-HDR- or ini-HDR-irradiation was performed, a

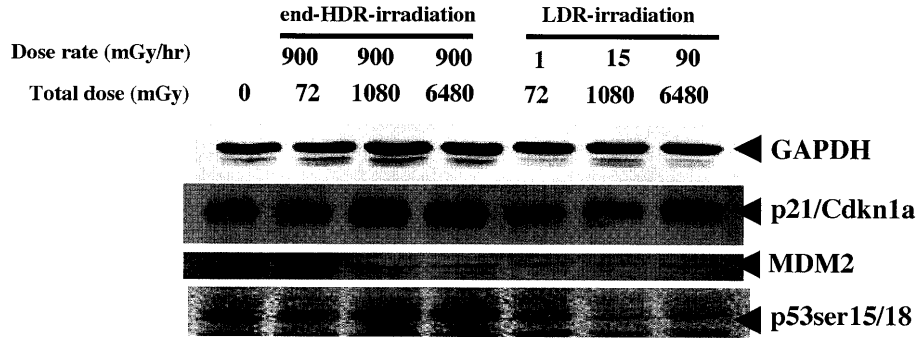


Fig. 4. Proteins expressions levels of p21/Cdkn1a, MDM2 and p53Ser15/18 in NIH/PG13Luc cells after beginning of LDR (1 mGy/hr, 15 mGy/hr, 90 mGy/hr) γ-ray irradiation for 72 hr. Gapdh was as an internal control. Total doses of 72 mGy, 1080 mGy, and 6480 mGy were used for irradiation experiments. Cells were treated with SDS sample buffer 4 hr after the end of irradiation.

different dose-rate-effect was observed compared with LDR-irradiation (Fig. 2B). Inverse dose-rate-effects were observed at total doses of more than 4320 mGy, when comparing LDR-irradiation with end-HDR irradiation, for the *Col1a2*, *Tnc*, and *Fbln5* genes.

Time-dependent gene expression following LDR-irradiation

To evaluate the relationshipsbetween the gene expression levels and total doses (irradiation time) in LDR-irradiation, the expression levels of the 5 genes were serially analyzed

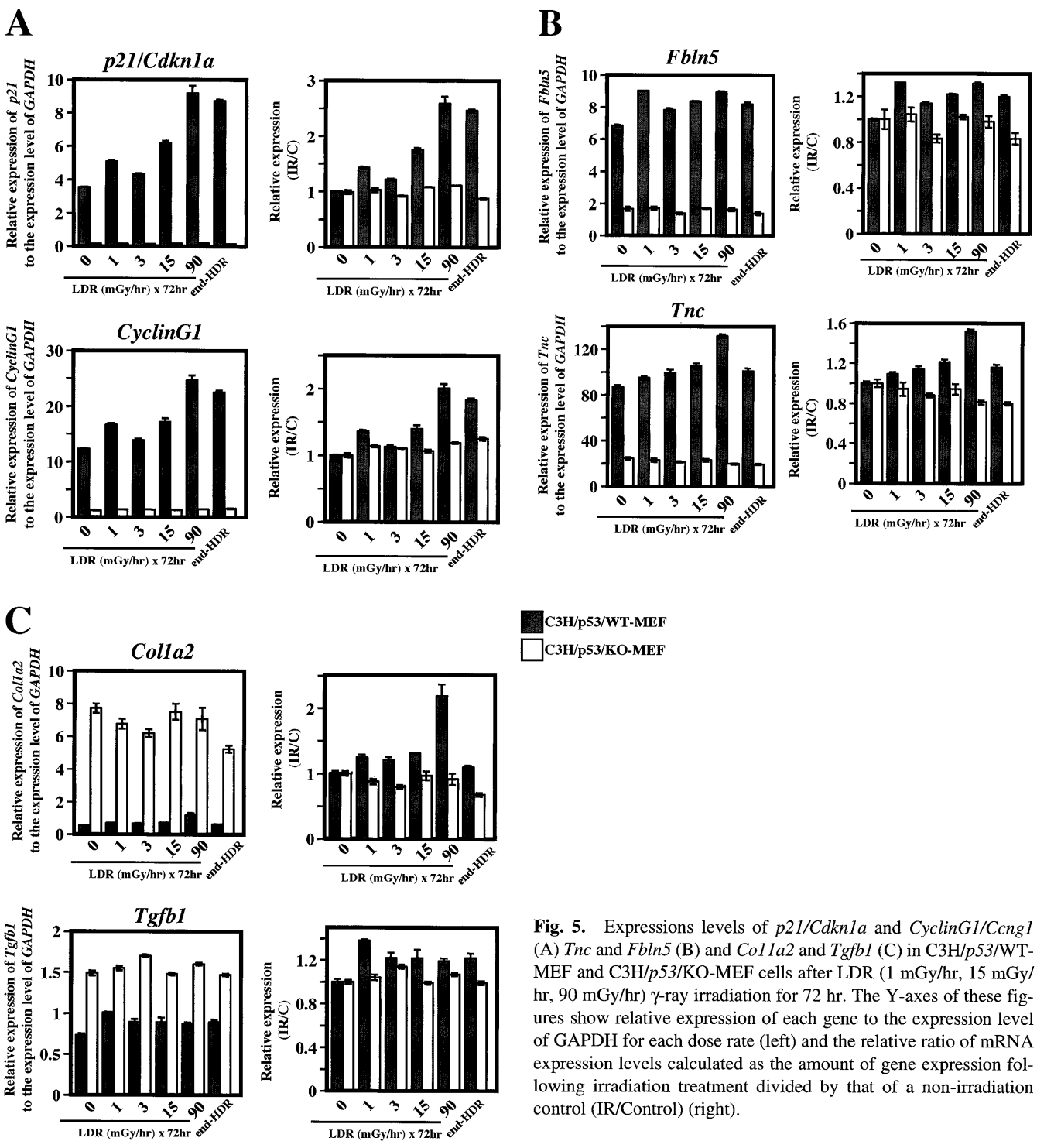


Fig. 5. Expressions levels of *p21/Cdkn1a* and *CyclinG1/Ccng1* (A) *Tnc* and *Fbln5* (B) and *Col1a2* and *Tgfb1* (C) in C3H/*p53*/WT-MEF and C3H/*p53*/KO-MEF cells after LDR (1 mGy/hr, 15 mGy/hr, 90 mGy/hr) γ-ray irradiation for 72 hr. The Y-axes of these figures show relative expression of each gene to the expression level of GAPDH for each dose rate (left) and the relative ratio of mRNA expression levels calculated as the amount of gene expression following irradiation treatment divided by that of a non-irradiation control (IR/Control) (right).

by real-time PCR at 24, 48 and 72 hr after the beginning of irradiation at dose-rates of 1, 15, and 90 mGy/hr (total doses of 24 mGy, 48 mGy, 72 mGy, 360 mGy, 720 mGy, 1080 mGy, 2160 mGy, 4320 mGy, and 6480 mGy, respectively). The expression levels of *Cdkn1a* and *Ccng1* increased from 24 hr, and their increase depended on both total dose and dose-rate in LDR-irradiation (Fig. 3A). On the other hand, the gene expression levels of *Col1a2*, *Tnc*, and *Fbln5* did not increase at 24 hr, but began to increase from 48 hr or 72 hr (Fig. 3B, Fig. 3C). Increase of the expression levels in these ECM genes did not depend on both total dose and dose-rate in LDR-irradiation. The expression of *Tgfb1* was also up-regulated at 72 hr in all LDR-irradiation treatments (Fig. 3C).

Protein expression following LDR- or HDR-irradiation

The amount of protein expression of Cdkn1a, MDM2, and p53ser15/18 was analyzed in LDR- (1, 15, and 90 mGy/hr for 72 hr) and end-HDR-irradiations (72, 1080, and 6480 mGy: 0.9 Gy/min) treatments (Fig. 4). The amount of Cdkn1a increased depending on total dose in all end-HDR-, ini-HDR-, and LDR-irradiation treatments, which showed a similar pattern to RNA expression obtained by real-time PCR analysis. As the stability of p53 is regulated by MDM2, which causes p53 degradation,[14,15] the protein expression of MDM2 was also analyzed. The amount of MDM2 decreased following end-HDR-irradiation at total doses of 1080 mGy and 6480 mGy, and also decreased by LDR-irradiation at 1, 15, 90 mGy/hr, comparing with that of the non-irradiated control. Interestingly, phosphorylated p53 at Ser15/18 did not increase after LDR-irradiation, or after ini-HDR irradiation (data not shown), although it increased in a dose-dependent manner up to 1080 mGy in the end-HDR-irradiation treatments.

Gene expression of p53knockout-MEF cells following LDR-irradiation

C3H/*p53*/KO-MEF cells irradiated by LDR- or HDR-irradiation showed almost no expression of *Cdkn1a* and *Ccng1* (Fig. 5A). Similarly, *Tnc* and *Fbln5* had almost no expression in C3H/*p53*/KO-MEF cells after both LDR- and HDR-irradiation (Fig. 5B). The expression levels of *Col1a2* and *Tgfb1* kept at high levels in C3H/*p53*/KO-MEF cells, but the LDR-irradiation induced an increase in expression of *Col1a2* and *Tgfb1* in C3H/*p53*/WT-MEF cells at 90 mGy/hr (Fig. 5C).

DISCUSSION

When end-HDR-irradiation or ini-HDR-irradiation were performed, different dose-rate-effects were observed compared with LDR-irradiation. The dose-rate-effects are generally found by comparing the biological effects induced by the same total dose at different dose-rates; however, when the timing of HDR-irradiation was changed, the effect on the dose-rate between LDR- and HDR-irradiation was also changed. This problem usually occurred when we estimated the dose-rate-effects of LDR-irradiation in irradiation experiments using mice as well as *in vitro* experiments.

The role of p53 in cellular responses to HDR-irradiation has been well investigated.[16] The present study confirmed enhanced expression levels of *Cdkn1a* and *Ccng1* by both LDR- and HDR-irradiations. Up-regulation of these genes following end-HDR- and ini-HDR-irradiation indicates immediate and delayed responses, respectively.

The expression levels of *Cdkn1a* and *Ccng1* in C3H/*p53*/KO-MEF cells were 20–50 fold lower than those in C3H/*p53*/WT-MEF cells in both LDR- and HDR-irradiation treatments. The result clearly demonstrated that the expression of *Cdkn1a* and *Ccng1* in ini-HDR-, end-HDR-, and LDR-irradiations treatments was mainly regulated by p53. The increase of gene expression of *Cdkn1a* and *Ccng1* by LDR-irradiation treatment was dependent on both total dose and dose rate. Interestingly, the present analysis showed no phosphorylation of p53 at Ser15/18 protein by LDR irradiation, although Cdkn1a protein increased similarly to the RNA expression of *Cdkn1a* in proportion to total dose. It was reported that the ataxia-telengestia-mutated (ATM) immediately responds to HDR-irradiation and activates phosphorylation of p53 at Ser15/18.[17] Therefore, the immediate expression of *Cdkn1a* and *Ccng1* in end-HDR-irradiation treatments is considered to be regulated by direct p53 transcriptional activity through ATM phosphorylation.[18] On the other hand, the phosphorylation of p53 at Ser15/18 activated by ATM may have a lower contribution to these expressions levels in ini-HDR or LDR-irradiation, because of the number of double strand breaks (DSBs) produced per unit time was low in LDR-irradiation. In present study, the protein expression levels of MDM2 were decreased comparing with that of non-irradiated control. Thus, enhanced gene expressions levels of *Cdkn1a* and *Ccng1* in ini-HDR- and LDR-irradiation treatments may be explained by lower degradation rates of p53, caused by low expression levels of MDM2.

Previous microarray analysis detected up- or down-regulated genes in response to LDR- (100 mGy/hr) irradiation.[11] Comparative analysis using microarray for gene expression levels at the same total doses (7200 mGy) between LDR- (100 mGy/hr) and end-HDR- (500 mGy/min) irradiations treatments identified genes with apparently different expression levels.[11] In the present re-calculation of the microarray results, highly expressed genes following LDR- (100 mGy/hr) irradiation included *Col1a2*, *Tnc*, and *Fbln5*, and up to 23.5% of these 34genes up-regulated genes was classified as ECM genes. Although, the same total dose was absorbed, the expression levels of *Col1a2*, *Tnc*, and *Fbln5* were different between ini-HDR and end-HDR-irradiations treatments. The expression time course of *Col1a2*, *Tnc*, and *Fbln5* genes after LDR-irradiation was similar to that in ini-

HDR-irradiation, and the expression profiles of *Col1a2*, *Tnc*, and *Fbln5* were clearly different from those of *Cdkn1a* and *Ccng1*. In addition, the expression of these genes began to increase from 48 or 72 hr after beginning of LDR-irradiation, indicating that the expression levels of these genes were not dependent on total dose or dose-rates. Inverse dose-rate-effects on the gene expression levels of *Tnc*, *Col1a2*, and *Fbln5*, found in the comparison of LDR-irradiation for 72 hr and end-HDR-irradiations, could be explained by the time of delayed responses to LDR-irradiations.

No enhancement of gene expression of *Tnc* and *Fbln5* was observed in either LDR- or HDR-irradiations in C3H/*p53*/KO-MEF cells, indicating that these genes seem to be regulated by p53. Similarly, the expressions levels of *Col1a2* and *Tgfb1* in C3H/*p53*/KO-MEF cells did not increase following LDR- or HDR-irradiation treatments, although they enhanced even in the non-irradiated control. Therefore, the expressions levels of *Col1a2* and *Tgfb1* following LDR- or HDR-irradiation also seems to be regulated by p53. However, *Col1a2*, *Tnc*, and *Fbln5* have not been reported to be activated by p53, as far as we know. HDR-irradiation induced p53 transcriptional activity and it was enhanced within 4 hr.[11] On the contrary, *Col1a2*, *Tnc*, and *Fbln5* showed delayed expression following LDR-irradiation. It is well known that gene expression down-stream of p53 cause cell cycle arrest or apoptosis.[19] Therefore, the observed such delayed expression found in *Col1a2*, *Tnc* and *Fbln5* might be result from the indirect effects of p53 such as cell cycle arrest or apoptosis, *etc.*

The promoter regions of *Col1a2* have an AP-1 binding site, regulated by TGF-β1,[20] and HDR-irradiation and LDR-irradiation induces TGF-β1.[7,21] Therefore, the up-regulation of *Col1a2* may be regulated through TGF-β1 produced in NIH/PG13Luc cells by LDR-irradiation. There are a few reports on TGF-β1 relating to *Tnc* or *Fbln5*, in which the up-regulation of *Tnc* in TGF-β1-stimulated human dermal fibroblasts involves a Smad3-mediated mechanism[22] and *Fbln5* plays a role in the regulation of cell growth and motility by affecting protein kinase activation by the addition of TGF-β1.[23] However, as we know, the relationship between expression levels of *Tnc* or *Fbln5* through TGF-β1 expressed by irradiation and activation of p53 has not been well studied.

Radiation induced-fibrosis is defined as a wound where continuous signals for tissue repair are emitted.[6] TGF-β1 is considered as a master switch for the formation of radiation induced-fibrosis.[6] Recently, Verrecchia F. *et al* reported that Smad3, which has a role at down stream of TGF-β1, relates with transcriptional regulation of collagen Type I gene and with development of fibrosis.[24] The p53 underlies the activation of radiation-induced plasminogen activator inhibitor-1 (PAI-1), which is thought to play major roles in the development of fibrosis along with the TGF-β/Smad pathway.[25] The relationship between the expression of *Col1a2*, *Tnc*, or *Fbln5* and fibrosis through TGF-β1 stimulated by LDR-irradiation remain to be clarified.

In conclusion, the inverse dose-rate effects on gene expressions levels of *Col1a2*, *Tnc*, and *Fbln5* were explained from the different time courses of expression following LDR- and HDR-irradiation. Furthermore, the present results on the little expression of the three ECM genes in LDR- and HDR-irradiation in p53-deficient cells suggest that these genes may be indirectly regulated by p53. Therefore, the two different time courses of gene expressions in LDR-irradiation treatments may be explained by the two different time-responses caused by the direct transcriptional activity of p53 related to early responses or by the indirect transcriptional activity of p53 with delayed responses, respectively.

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