Integrative genomic and proteomic analyses identifies glycerol-3-phosphate acyltransferase as a target of low-dose ionizing radiation in EBV infected-B cells

Yoko Tabe\textsuperscript{a,i}, Yasuhito Hatanaka\textsuperscript{a}, Mayumi Nakashiro\textsuperscript{b}, Kazumasa Sekihara\textsuperscript{a,k}, Shinichi Yamamoto\textsuperscript{a,k}, Hiromichi Matsushita\textsuperscript{c}, Saiko Kazuno\textsuperscript{d}, Tsutomu Fujimura\textsuperscript{d,e}, Takako Ikegami\textsuperscript{f}, Keita Nakanaga\textsuperscript{g}, Hirotaka Matsumoto\textsuperscript{b}, Takashi Ueno\textsuperscript{d}, Junken Aoki\textsuperscript{g}, Takehiko Yokomizo\textsuperscript{b}, Marina Konopleva\textsuperscript{i}, Michael Andreeff\textsuperscript{i}, Takashi Miida\textsuperscript{a}, Kazuhisa Iwabuchi\textsuperscript{j} and Keisuke Sasai\textsuperscript{b}

\textsuperscript{a}Department of Laboratory Medicine, Juntendo University, Tokyo; \textsuperscript{b}Department of Radiology, Juntendo University, Tokyo; \textsuperscript{c}Department of Laboratory Medicine, Tokai University School of Medicine, Kanagawa; \textsuperscript{d}BioMedical Research Center, Juntendo University, Tokyo; \textsuperscript{e}Laboratory of Bioanalytical Chemistry, Tohoku Pharmaceutical University, Miyagi; \textsuperscript{f}Laboratory of Molecular and Biochemical Research, Research Support Center, Juntendo University, Tokyo; \textsuperscript{g}Graduate School of Pharmaceutical Sciences, Tohoku University, Miyagi; \textsuperscript{h}Department of Biochemistry (I), Juntendo University, Tokyo, Japan; \textsuperscript{i}Department of Leukemia, the University of Texas MD Anderson Cancer Center, Houston, Texas, USA; \textsuperscript{j}Research Institute for Environmental and Gender Specific Medicine, Juntendo University, Tokyo, Japan; \textsuperscript{k}Leading Center for the Development and Research of Cancer Medicine, Juntendo University, Tokyo, Japan

\textbf{ABSTRACT}

\textbf{Purpose:} We sought to gain a better understanding of the low-dose ionizing radiation (LDIR)-induced molecular changes in transformed pre-malignant cells in their microenvironment.

\textbf{Materials and methods:} The cellular response to LDIR was compared and contrasted using immortalized human Epstein-Barr virus-infected B-cells (EBV-B) in mono-culture, co-culture with human bone marrow derived stromal cells (MSC), or under the LDIR-induced bystander effect. The resulting alterations in protein and gene expression (including microRNA, miRNA) were evaluated by isobaric tags for relative and absolute quantification (iTRAQ) proteomics assay, western blot, cDNA array and quantitative reverse transcription polymerase chain reaction (RT-PCR), respectively.

\textbf{Results:} The miRNAs \textit{let7a}, \textit{miR-15b}, \textit{miR-16}, and \textit{miR-21}, and a lipid metabolic miRNA hub \textit{miR-23b}, were upregulated after LDIR exposure in the mono-cultured EBV-B cells, but were downregulated in EBV-B cells co-irradiated with MSC. A lipid biosynthesis enzyme glycerol-3-phosphate acyltransferase, the common target of these miRNA, was downregulated at the level of protein and mRNA expression in the LDIR-exposed, mono-cultured EBV-B cells and upregulated MSC co-cultured EBV-B cells.

\textbf{Conclusions:} These results suggest a putative miRNA regulatory mechanism controlling the LDIR-induced stress response, and illustrate that LDIR exposure, and the cell’s microenvironment, can affect specific gene expression, both directly and indirectly, resulting in altered protein expression.

\textbf{Introduction}

Ionizing radiation is known to induce remodeling of the stromal microenvironment, and the carcinogenic potential of this environmental stressor is well documented (Allan and Travis 2005). Typically, ionizing radiation exposures occur in the low-dose range ($\leq$ 100 milligray, [mGy]). The biological effects of low-dose ionizing radiation (LDIR) have long been investigated, but they are still relatively ambiguous (Morgan and Sowa 2009). Hence, it is important to determine the deleterious health consequences of LDIR in relation to potential exposures that are as varied as those associated with healthcare, military operations, space exploration, and energy production. This is especially true with respect to the potential for large-scale environmental exposures such as those that occurred after the nuclear power plant catastrophes at Chernobyl in 1986 (Saenko et al. 2011) and Fukushima Daiichi in 2011.

Indeed, emergency workers received doses of roughly 100 mGy or greater following the Fukushima Daiichi disaster, and they still undergo routine monitoring for potential delayed radiation-related health effects. Furthermore, contaminated areas vicinal to the Fukushima Daiichi plant remain a considerable concern because of the potential for exposure to LDIR (Akiha 2012).

Biologic responses to LDIR are commonly described as a stress response, which include adaption, bystander effects, genomic instability, and LDIR hypersensitivity. As a mechanism of adaption, LDIR can provide some degree of cells resistance to genetic mutations and chromosomal aberrations by subsequent exposures to radiation or other genotoxic agents (Wolff et al. 1988, SASAI et al. 2002), which is known as the radioadaptive response (Pant et al. 2003). The activation of DNA repair pathways is intimately linked with other cellular...
pathways, including transcription, cell-cycle checkpoint arrest, and apoptosis (Chaudhry 2008), such that irradiated cells respond appropriately to being either repaired or eliminated via apoptosis. Indeed, DNA double-strand break (DSB) repair pathway activation and its epigenetic memory by histone marking have been reported to be implicated in a unique tissue response to LDIR (Sasai et al. 2014). Bystander effects are the signaling interactions of an irradiated cells with neighboring cells (Prise and O’Sullivan 2009), and this has been observed in various cell types including lymphocytes (Kadhim et al. 2004), fibroblasts (Prise et al. 1998), and tumor cells (Shao et al. 2002). microRNAs (miRNA) are a large family of small non-coding RNAs of 18-24 nucleotides that generally act as negative post-transcriptional gene regulators (Nilsen 2007). A single miRNA has broad effects on gene expression networks, including those of stress responses (Marsit et al. 2006), and miRNA acts as hub regulators of specific cellular responses and mediate signaling pathways linked to cancer development (Maes et al. 2008). While miRNA has been reported to be involved in cell response to LDIR (Marsit et al. 2006), its functions have not been well studied.

Epstein-Barr virus (EBV), a prototypic human tumour herpesvirus, is carried by the majority of adults as an asymptomatic infection, being associated with several malignancies, including B- and T-cell lymphomas, nasopharyngeal carcinoma, and some gastric carcinomas (Calderwood et al. 2007). EBV is known for its capacity to deregulate B-lymphocyte growth by constitutively activating pathways that trigger cellular gene expression (Henderson et al. 1991). This activity is linked to the development of Burkitt’s lymphoma (Epstein et al. 1964) and other B-cell lymphomas (Purtito et al. 1982, Hanto et al. 1983).

To gain insights into the molecular changes induced by LDIR in EBV-infected, continuously-proliferating lymphoblastoid B-cells that normally reside in periphery or in the lymph nodes, we utilized an in vitro co-culture system using human immortalized, EBV–transformed, B-lymphocytes (EBV-B) and human stromal cells.

Proteomic analysis by isobaric tags for relative and absolute quantitation (iTRAQ) presented some preliminary screen of protein expression changes, including the bystander effect, in mediating a novel pathway associated with the LDIR cellular stress responses. We further report parallel investigations to profile the expression of messenger RNA (mRNA) and miRNA acutely altered by LDIR exposure, which revealed the putative miRNA-associated regulation of the mitochondrial enzyme glycerol-3-phosphate acyltransferase (GPAM) expression in the EBV-immortalized B-cells.

Materials and methods
Cell lines, primary samples, and culture conditions
The EBV-immortalized B-cells (EBV-B) (Danjoh et al, 2012) were purchased from Health Science Research Resources Bank (Sennan, Osaka, Japan); these cells were cultured alone or co-cultured with MSC in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2% fetal bovine serum (FBS, all purchased from Sigma-Aldrich, St. Louis, MO, USA). MSC from healthy bone marrow donors were obtained after informed consent was obtained in accordance with institutional guidelines set forth by The University of Texas MD Anderson Cancer Center per Declaration of Helsinki principles. The MSC was cultured at a density of 20,000 cells/cm² in minimum essential medium alpha (MEMα) supplemented with 20% heat-inactivated FBS, 1% L-glutamine, and 1% penicillin-streptomycin at 37°C in 5% CO₂ (Sigma-Aldrich). The cultured MSC comprised a single phenotypic population, as determined by flow cytometric analysis, positive for SRC Homology 2 and 3 Domain (SH2 and SH3) and negative for markers of hematopoietic lineage. MSC harvested after passage 3 or 4 were used for the co-culture experiments.

For LDIR exposure, both types of cells were irradiated with 100 mGy or 1 Gy in mono- or co-culture conditions at room temperature. All irradiations were carried out with a 4 megavolt (MeV) X-ray generated by a medical Linac (Clinac 21EX, Varian, Palo Alto, CA, USA) following full build-up (1 cm) at a dose rate of 2.0 Gy/min, as previously reported (Sasai et al. 1990).

The cells were then harvested at indicated times. Two main groups of EBV-B cells under mono-culture (moB) and co-culture (coB) conditions were prepared. The group of EBV-B cells co-cultured with MSC (coB) contained two subgroups, directly irradiated EBV-B cells (coB-IR), and bystander EBV-B cells, which were co-cultured with pre-irradiated MSC (coB-BS). For bystander coB-BS cells, sham irradiated EBV-B cells were co-cultured with irradiated MSC immediately following the irradiation of MSC. Each main group has distinct controls; mono-cultured EBV-B cells without irradiation (moB-cont) for moB-IR cells and EBV-B cells co-cultured with MSC without irradiation (coB-cont) for coB-IR and coB-BS cells. MSC without or with 100 mGy irradiation were also prepared (Figure 1).

Co-cultured EBV-B cells were separated from the MSC monolayer by careful pipetting with ice-cold phosphate-buffered saline solution, repeated twice. After the EBV-B cells were collected, we confirmed that they did not express thymus cell antigen-1 (CD90) mRNA by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to exclude contamination by MSC.

Cell-cycle analysis
Cell cycle distribution was assessed by flow cytometry of propidium iodide-stained nuclei (Milella et al. 2001). Cells were fixed in ice-cold ethanol (70% vol/vol) and stained with propidium iodide solution (25 μg/ml propidium iodide, 180 U/ml RNase, 0.1% Triton X-100, and 30 mg/ml polyethylene glycol in 4 mM citrate buffer, pH 7.8; Sigma-Aldrich). DNA content was determined by FACScan flow cytometer and CellQuest acquisition and analysis programs. Gating was set to exclude cell debris, cell doublets, and cell clumps.

iTRAQ sample labelling, mass spectrometry analysis, and peptide identification
Analysis of proteins by isobaric tags for relative and absolute quantification (iTRAQ), a chemical label detected by mass spectrometry, was performed as described previously.
Cell lysate samples were concentrated and buffer exchanged using 3.5-kDa molecular weight cut-off spin concentrators (Tomy Seiko Co., Ltd, Tokyo, Japan), then digested for 24 h with 10 μg L⁻¹-(4-tosylamido)-2-phenylethyl tosylphenylalanyl chloromethyl ketone-treated trypsin. Each peptide solution was labelled with iTRAQ reagents according to the manufacturer’s protocol (AB SCIEX, Framingham, MA, USA). Labelled peptides were pooled and fractionated by strong cation exchange, using a ChromXP C18-CL column (Eksigent parts of AB SCIEX, Dublin, California, USA), and analyzed by nano liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS); nano LC-MS was performed on a TripleTOF 5600 mass spectrometer for MS/MS (AB SCIEX) interfaced with a nano LC system (AB SCIEX) (Glen et al. 2008). Protein identification and relative quantification was carried out by applying ProteinPilot Software Version 4.5 (AB SCIEX) to the data as previously described (Evans et al. 2012).

The functions of the various protein contents were searched against the Swissport database (release10/16/2013) using the search algorithm within the ProteinPilot Software and the Analyst TF Software (AB SCIEX). Protein ratios were normalized by using the overall median ratio for all the peptides in the sample for each separate ratio in every individual experiment. Two independent iTRAQ experiments were carried out to profile and quantitate the proteome and the three replicate samples were used to determine the cut-off for significant fold-change. A confidence cut-off of >95% was applied for protein identification, and >1.2-fold change cut-off for all iTRAQ ratios was selected to classify proteins as up- or down-regulated as previously described (Datta et al. 2010). Candidate proteins that commonly changed in the same direction (i.e., up or down) in two experimental samples were used for pathway analysis by Metacore (GeneGo, St. Joseph, MI, USA), which represents interactions compiled from a curated database of human protein interactions, metabolism, and bioactive compounds (Ekins et al. 2006).

cDNA microarray

A microarray analysis was performed by using the Affymetrix Human Gene 1.0 ST Array according to Affymetrix protocols (Santa Clara, CA, USA). Signal intensities were measured by using a GeneChip Scanner3000 (Affymetrix) and converted to numerical data by using the GeneChip Operating Software, version 1 (Affymetrix). The genes whose expression levels were changed by more than 1.6-fold after irradiation were defined as ‘profoundly modulated genes’, as previously described (Taki et al. 2009). The digitized data were analyzed by GeneSpring 3.2.2 software (Silicon Genetics, Redwood, CA, USA).

Validation of the microarray data by quantitative real-time RT–PCR

Reverse transcription polymerase chain reaction (RT-PCR) analyses of triplicate samples were conducted for the validation of gene expression levels and screened by a cDNA microarray. Total RNA was extracted from cells with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis was performed with oligo (dT) as primer (Superscript II System; Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed by using the Model 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). mRNA expressions coding glycerol-3-phosphate acyltransferase (GPAM), amphiregulin (AREG), Interleukin 8 (IL-8), formin 1 (FMN1), B-cell...
lymphoma 2 (BCL2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was detected by TaqMan Gene Expression Assays (GPAM: Hs01573680_m1; AREG: Hs00950669_m1; IL-8: Hs00174103_m1; FN1: Hs02384610_m1; BCL2: Hs00608023_m1; GAPDH: Hs99999905_m1; Applied Biosystems). The PCR cycle number that generated the first fluorescence signal above the threshold cycle value (Ct) was determined. The abundance of each gene transcript relative to that of GAPDH was calculated as follows: Relative expression \( = 100 \times 2^{-\Delta\Delta Ct} \), where \( \Delta C_t \) was the mean \( C_t \) of the transcript of interest minus the mean \( C_t \) of the transcript for GAPDH. The \( C_t \) data from duplicate PCR reactions were averaged for calculation of relative expression.

**RNA extraction and miRNA RT-PCR analysis**

For miRNA qRT-PCR assay, RNA was extracted with an miRNeasy Mini Kit (Qiagen). Mature miRNA let-7a, miR-16, miR-19b, miR-21, and miR-27b were quantified by using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) utilizing the Model 7500 Real-time PCR System (Applied Biosystems). Expression of let-7a, miR-16, miR-19b, miR-21, miR-27b and U6 snRNA was detected by TaqMan MicroRNA Assays (hsa-let-7a: 000377; hsa-miR-16: 000391; hsa-miR-19b: 000396; hsa-miR-21: 000397; hsa-miR-27b: 000409; U6 snRNA: 001973; Applied Biosystems). The PCR cycle number that generated the first fluorescence signal above a threshold value (the threshold cycle; \( C_t \)) was determined. The abundance of each transcript of miRNA relative to that of U6 snRNA was calculated. The \( C_t \) data from duplicate PCR reactions were averaged for calculation of relative expression. Three independent experiments were carried out. miRNA targets were predicted by using an algorithm of the mircoRNA.org (http://www.microrna.org/microrna/home.do) website tools.

**Western blot analysis**

Cells were subjected to solubilization in lysis buffer (phosphate-buffered saline solution, \( 1 \times \) cell lysis buffer [Cell Signaling Technology, Beverly, MA, USA], \( 1 \times \) protease inhibitor [Roche, Indianapolis, IN, USA], and \( 1 \times \) phosphatase inhibitor cocktails I and II [Calbiochem, San Diego, CA, USA]) and then incubated for 30 min on ice. The lysates were subjected to centrifugation for 10 min 20,000 g at 4°C, and the supernatants were further analyzed. Total protein concentration was determined by the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s protocol. Samples of the total protein (40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories) and transferred to a polyvinylidene fluoride membrane (0.45 μm, GE Healthcare, Buckinghamshire, UK), then probed with first and second antibodies according to the manufacturers’ protocols. For immunoblotting, α-tubulin (Sigma-Aldrich), GPAM (Abcam, Cambridge, MA, USA), and horse radish peroxidase-linked anti-mouse and anti-rabbit Immunoglobulin G (both from Cell Signaling Technology, Danvers, MA, USA) were used. Three independent experiments were performed.

**Quantification of phospholipids by LC-MS/MS**

Our liquid chromatography-mass spectroscopy (LC-MS/MS) system consisted of a NANOSPACE SI-2 HPLC (Shiseido, Chuo-ku, Tokyo, Japan) and a TSQ Quantum Ultra triple quadropole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a heated electrospray ionization (ESI) source. For diacyl phospholipid analysis, lipids were extracted from cells (\( 1 \times 10^6 \)) by suspending them in 100 μl 1-butanol containing internal standards 12:0/12:0 phosphatidylglycerol (PG), 12:0/12.0 phosphatidylethanolamine (PE), 12:0/12:0 phosphatidylycerine (PS), and 14:0/14:0 phosphatidylcholine (PC). The extracts were subjected to sequential centrifugation, and the resulting supernatants were analyzed after filtration. Lipids were separated by a SILILCA SG80 column (2.0 × 150 mm i.d., 5 μm particle size, Shiseido), using a gradient of solvent A (5 mM ammonium formate in water) and solvent B (acetoni trile). The initial condition was set at 100% B.

The following solvent gradient was applied: a 15-min gradient to 82.5% B, a 2-min gradient to 30% B, hold 30% B for 2 min, followed by re-equilibration of the column by a 3-min gradient to 100% B and a 8-min hold at 100% B. The flow rate was set at 400 μl/min, PC, PE and PS were analyzed in the positive ion mode. PC-containing species were discriminated by measuring the precursors of m/z 184. PE-containing species were detected by scanning for a neutral loss of 141 Da. The PS-containing species were detected by scanning for molecules that underwent a neutral loss of 185 Da. PG and phosphatidylinositol (PI) and were quantified by full scans in the negative ion mode. The ratio between analyte and internal standard peak area was used for quantification.

**Statistical analyses**

Unless otherwise indicated, differences between groups of cells were analyzed by the two-tailed Student’s t-test. A difference was considered statistically significant when \( p < 0.05 \). Average values were expressed as mean ± standard deviation (SD) of triplicate samples unless otherwise mentioned (Figure 1).

**Results**

**The anti-apoptotic effect of MSC on EBV-B cells**

To confirm the ability of the co-culture system to mimic the microenvironment of the lymph node where proliferating lymphoblastoid B-cells normally reside, we first examined the cytoprotective effect of MSC on EBV-B cells. Flow cytometric analysis of propidium iodide-stained cell nuclei showed that spontaneous apoptosis along with a G1 phase accumulation of EBV-B cells following serum starvation (Figure 2).

**The proteome of EBV-B is modulated by LDIR**

To assess protein expression changes by LDIR, and to identify the pathways involved in the LDIR effects in EBV-B, as well as
MSC, we then employed iTRAQ proteomics analysis to exhaustively analyze expression profiles following acute irradiation (Figure 1). iTRAQ detected a total of 2257 and 2212 unique proteins in the two independent experiments.

Pathway analysis (both at 1- and 24-h time-points) revealed that the mono-cultured EBV-B cells exposed to LDIR exhibited continuously upregulated proteins associated with protein folding and proteasomal degradation. This was accompanied by the upregulation of the DNA replication licensing factor and DNA damage-binding protein, and activation of metabolic pathways after 24 h of culture (Table IA and Supplementary Table IA, available online with all Supplementary material). In monocultured MSC, LDIR upregulated keratins, fibrillin, myosins, actinins, and vimentin, as well as elongation factor and glucose-6-phosphate-1 dehydrogenase at 1 h and/or 24 h (Supplementary Table IB).

Interestingly, as an apparent bystander effect the upregulation of gene transcription- and ribosomal biogenesis-associated proteins, including ribosomal proteins, a eukaryotic initiation factor, nucleolar phosphoprotein nucleolin, and ATP synthase, was observed in EBV-B cells under 1 hour co-culture with irradiated MSC (Supplementary Table IC). As shown in Table I, cell cycle and apoptosis pathways were also altered by LDIR, consistent with the previous reports (Ishikawa et al. 2006, Yue et al. 2009). We further observed that the alteration of several pathways including cell adhesion and cytoskeleton remodeling overlapped extensively among different culture conditions and time-points.

Notably, iTRAQ proteomics analysis detected upregulation of cell surface glycoprotein cluster of differentiation 44 (CD44) phosphorylation on constitutive phosphorylation site Ser 706 in irradiated EBV-B alone (1.3-fold) as well as the bystander EBV-B (1.7-fold). CD44 is involved in cell-cell interactions, cell adhesion, and migration (Misra et al. 2011). Alteration of immune response signaling was frequently observed in the co-irradiated cells and bystander EBV-B but not in the irradiated EBV-B cultured alone. These results suggested that the signaling pathways activated by LDIR in EBV-B differed in the presence or absence of stromal cells (Figures 3 and 4).

**LDIR-modulates gene expression in EBV-B cells**

We next investigated alterations in gene expression after acute (24-h) exposure to LDIR in EBV-B using the Affymetrix cDNA microarray platform (Figure 1). In mono-cultured EBV-B cells, cDNA microarray analysis showed that LDIR upregulated 1 gene and downregulated 8 genes (>1.6-fold change). We observed the downregulation of the genes encoding a lipid biosynthesis enzyme glycerol-3-phosphate acyltransferase (GPAM) and a growth factor amphiregulin (AREG) (Supplementary Table II), which are known to be negatively regulated by transforming growth factor beta (TGFβ) (Bennett et al. 1992). LDIR downregulated cell survival-/growth-related factors Rho-GTPase effector gene fomlin-1 (FMN1) (Supplementary Table I), chemotaxis-inducing chemokine gene IL-8 (1.33-fold) and B-cell lymphoma 2 gene BCL2 (1.58-fold), which were confirmed by qRT-PCR (Figure 3A). Downregulation of the GPAM protein was detected by Western blot (Figure 3B).

In MSC co-culture condition, irradiation profoundly modulated expression of six genes (three upregulated and three downregulated, >1.6-fold change) in EBV-B cells compared to the controls, including the upregulation of the genes encoding transmembrane protein (TMEM) and olfactory receptor (OR2H) (Supplementary Table II). In contrast to the mono-culture condition, the GPAM mRNA and protein expression were upregulated in EBV-B cells co-irradiated with MSC (Figure 3B and 4).

As an indicator of the bystander effect, we detected the upregulation of the serpin peptidase inhibitor gene (SERPINB2), which are related to the invasive potential of cancer cells (Liu et al. 2013) (Supplementary Table II).

**LDIR-modulated miRNA changes in EBV-B**

Recently, ionizing radiation-induced alterations in miRNA expression have been demonstrated in various cell types (Chaudhry et al. 2010b, Evans et al. 2012). To identify LDIR-induced modulation of specific miRNA in EBV-B cells, we assembled a high-confidence list of 44 known LDIR-associated miRNA from three screen studies of lymphoblastic cells: (1) a large-scale miRNA expression profiling (microarray) in response to LDIR (Cha et al. 2009); (2) a systematic miRNA screen for LDIR induced alteration assayed by quantitative RT-PCR analysis (Chaudhry et al. 2010a); and (3) a comprehensive study by analyzing previously reported dataset of miRNA modulated in low-dose irradiated cells (Lhakhang and Chaudhry 2012). We then selected four miRNA, let-7a, miR-16, miR-19b, and miR-21, which have been listed in more than two of these studies, and their expression changes in EBV-B cells under different culture conditions were investigated. LDIR upregulated let-7a and miR-16 levels in irradiated EBV-B cultured alone compared to the controls and downregulated all tested miRNA under the co-irradiation condition with MSC (Figure 5A).

Figure 2. Co-culture with MSC inhibited spontaneous apoptosis of EBV-B cells. EBV-B cells were cultured under serum starved condition (FCS 0%) with or without MSC co-culture. After 48 and 72 h, the percentage of apoptotic cells (sub-G1) were detected by propidium iodide flow cytometry. Graphs show the mean ± SD of results of three independent experiments. *p < 0.05, **p < 0.01.
### (A) Pathway analysis of proteins significantly altered by LDIR in mono-cultured EBV-B cells (1-hour and 24-hour time-points).

| LDIR condition | (time point) | GeneGo Pathway Maps | p Value |
|----------------|--------------|---------------------|---------|
| mono-cultured EBV-B cells (moB-IR vs moB-cont) | (1 hour) | Cell adhesion_Role of CD5 in cell adhesion | 9.82E-03 |
| | | Proteolysis_Putative ubiquitin pathway | 2.49E-02 |
| | | Cell adhesion_Endothelial cell contacts by non-junctional mechanisms | 2.60E-02 |
| | | CFTR folding and maturation (normal and CF) | 2.60E-02 |
| | | Development_Glucocorticoid receptor signaling | 2.60E-02 |
| | | Proteolysis_Role of Parkin in the Ubiquitin-Proteasomal Pathway | 2.60E-02 |
| | | Cell adhesion_Cadherin-mediated cell adhesion | 2.81E-02 |
| | | Development_STP2 and STP3 receptors in cell proliferation and differentiation | 2.81E-02 |
| | | Cell adhesion_Endothelial cell contacts by junctional mechanisms | 2.81E-02 |
| | | Transcription_Role of Akt in hypoxia induced HIF1 activation | 2.92E-02 |
| | (24 hour) | CFTR folding and maturation (normal and CF) | 1.84E-04 |
| | | Apoptosis and survival_Granzyme A signaling | 3.62E-04 |
| | | Regulation of degradation of deltaS508-CFTR in CF | 7.91E-04 |
| | | ATP metabolism | 1.50E-03 |
| | | GTP metabolism | 2.04E-03 |
| | | Transport_RAN regulation pathway | 3.16E-03 |
| | | dGTP metabolism | 3.47E-03 |
| | | DNA damage_NHEJ mechanisms of DSBs repair | 3.52E-03 |
| | | TTP metabolism | 3.63E-03 |
| | | LRRK2 and immune function in Parkinson's disease | 4.72E-03 |

Proteins that showed significant expression changes (p < 0.05) compared to controls were selected. The top 10 pathway maps identified by metacore in the iTRAQ data sets of Supplementary Table IA for each condition are shown.

### (B) Pathway analysis of proteins significantly altered by LDIR in mono-cultured MSC (1-hour and 24-hour time-points).

| LDIR condition | (time point) | GeneGo Pathway Maps | p Value |
|----------------|--------------|---------------------|---------|
| mono-cultured MSC (MSC-IR vs MSC-cont) | (1 hour) | Cell adhesion_Role of CD5 in cell adhesion | 9.82E-03 |
| | | Proteolysis_Putative ubiquitin pathway | 2.49E-02 |
| | | Cell adhesion_Endothelial cell contacts by non-junctional mechanisms | 2.60E-02 |
| | | CFTR folding and maturation (normal and CF) | 2.60E-02 |
| | | Development_Glucocorticoid receptor signaling | 2.60E-02 |
| | | Proteolysis_Role of Parkin in the Ubiquitin-Proteasomal Pathway | 2.60E-02 |
| | | Cell adhesion_Cadherin-mediated cell adhesion | 2.81E-02 |
| | | Development_STP2 and STP3 receptors in cell proliferation and differentiation | 2.81E-02 |
| | | Cell adhesion_Endothelial cell contacts by junctional mechanisms | 2.81E-02 |
| | | Transcription_Role of Akt in hypoxia induced HIF1 activation | 2.92E-02 |
| | (24 hour) | Cell adhesion_Endothelial cell contacts by junctional mechanisms | 7.32E-06 |
| | | Cell adhesion_Chemokines and adhesion | 1.25E-05 |
| | | Cytoskeleton remodeling_Cytoskeleton remodeling | 1.35E-05 |
| | | Cytoskeleton remodeling_Keratin filaments | 1.99E-05 |
| | | Cytoskeleton remodeling_Integrin outside-in signaling | 5.08E-05 |
| | | Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling | 5.78E-04 |
| | | Development_Glucocorticoid receptor signaling | 5.92E-04 |
| | | Cell adhesion_Endothelial cell contacts by non-junctional mechanisms | 5.92E-04 |
| | | CFTR folding and maturation (normal and CF) | 5.92E-04 |
| | | Cytoskeleton remodeling_Neurolaminins | 6.42E-04 |

Proteins that showed significant expression changes (p < 0.05) compared to controls were selected. The top 10 pathway maps identified by metacore in the iTRAQ data sets of Supplementary Table IB for each condition are shown.

### (C) Pathway analysis of proteins significantly altered by LDIR in EBV-B cells co-cultured with MSC after exposure to LDIR (1-hour and 24-hour time-points).

| LDIR condition | (time point) | GeneGo Pathway Maps | p Value |
|----------------|--------------|---------------------|---------|
| EBV-B co-culture with MSC (coB-IR vs coB-cont) | (1 hour) | Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases | 1.00E-08 |
| | | Transport_The role of AYP in regulation of Aquaporin 2 and renal water reabsorption | 2.56E-07 |
| | | Cytoskeleton remodeling_Cytoskeleton remodeling | 4.57E-06 |
| | | Development_SLIT-Robo signaling | 5.20E-06 |
| | | Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling | 6.40E-06 |
| | | Development_MAG-dependent inhibition of neurite outgrowth | 9.90E-06 |
| | | Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK. | 1.98E-05 |
| | | Cell adhesion_Integrin outside-in signaling and migration | 2.19E-05 |
| | | Immune response__CCR3 signaling in eosinophils | 9.08E-05 |
| | | wtCFTR and deltaS508-CFTR traffic / Clathrin coated vesicles formation (normal and CF) | 2.48E-04 |
| | (24 hour) | Glycolysis and gluconeogenesis p.3 | 8.80E-04 |
| | | Glycolysis and gluconeogenesis p.3 | 8.80E-04 |
| | | Signal transduction cAMP signaling | 2.21E-03 |
| | | Regulation of lipid metabolism_Regulation of lipid metabolism by niacin and isoprenaline | 3.08E-03 |
| | | PTMs in BAF7-induced signaling | 3.95E-03 |
| | | Apoptosis and survival_Role of PRK in stress-induced apoptosis | 4.26E-03 |
| | | Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK. | 1.98E-05 |
| | | Cell adhesion_Integrin outside-in signaling and migration | 2.19E-05 |
| | | Immune response__CCR3 signaling in eosinophils | 9.08E-05 |
| | | wtCFTR and deltaS508-CFTR traffic / Clathrin coated vesicles formation (normal and CF) | 2.48E-04 |

Proteins that showed significant expression changes (p < 0.05) compared to controls were selected. The top 10 pathway maps identified by metacore in the iTRAQ data sets of Supplementary Table IC for each condition are shown.

(bystander EBV-B of LDIR MSC (coB-BS vs coB-cont) | (1 hour) | Cytoskeleton remodeling_Cytoskeleton remodeling | 1.32E-04 |
| | | Oxidative phosphorylation | 1.47E-04 |
| | | Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases | 1.62E-03 |
| | | Proteolysis_Role of Parkin in the Ubiquitin-Proteasomal Pathway | 1.77E-03 |
| | | Cytoskeleton remodeling_Neurolaminins | 1.92E-03 |
| | | Cell cycle_Initiation of mitosis | 1.92E-03 |
| | | Translation_Regulation of translation initiation | 2.24E-03 |
| | | Cell cycle_Role of Nek in cell cycle regulation | 3.13E-03 |
| | | Apoptosis and survival_Regulation of Apoptosis by Mitochondrial Proteins | 3.33E-03 |
| | | Cytoskeleton remodeling_Keratin filaments | 3.95E-03 |

(continued)
To narrow down the targets of LDIR-altered miRNA, we extracted 79 genes that are commonly targeted by let-7a, miR-16, miR-19b, or miR-21 identified from a microrna.org database (Supplementary Table III). Among the predicted common target mRNAs, the expression changes of GPAM mRNA in response to LDIR detected by qRT-PCR and microarray as well as protein levels detected by Western blot were strikingly matched with these results. Partial concordant changes of DNAJ (HSP40) A2 gene coding heat shock protein (HSP) 40 homolog, a co-chaperone of HSP70s (Takayama et al. 2003), CPEB3 gene coding RNA binding protein cytoplasmic polyadenylation element binding protein 3 (CEBP3) and its transcriptional target glutamate receptor subunit (GLUR2) gene, a receptor of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (Huang et al. 2006), were observed in the microarray results.

Since GPAM is known to be a lipid-metabolism gene (Gonzalez-Baro et al. 2007, Teslovich et al. 2010) which has been reported to be regulated by miR-27b, a candidate of regulatory hub in lipid metabolism (Vickers et al. 2013), we investigated the expression changes of GPAM mRNA after LDIR in EBV-B cells. As expected, LDIR upregulated miR-27b in monolayer EBV-B cells, and downregulated in co-cultured EBV-B cells with MSC (Figure 5B).

To clarify whether the expression changes of GPAM mRNA, as well as miR-27b, were LDIR-specific, we investigated the responses again after higher dose, 1 Gy, irradiation. The 1 Gy exposure did not induce significant changes of GPAM mRNA or miR-27b in the monolayer or cocultured EBV-B cells, suggesting that these effects were specific to LDIR (Figures 3B and 5B).

GPAM plays a key role in lipid biosynthesis as the rate-limiting enzyme that catalyzes the first committed step in de novo triglyceride synthesis (Gonzalez-Baro et al. 2007) and in phospholipid biosynthesis (Teslovich et al. 2010). Phosphatidylcholine (PC), the major composition of glycerol-
based phospholipids that are the main lipid component of biological membranes, has been reported to be regulated by GPAM activity in breast cancer (Brockmöller et al. 2012). In EBV-B cells, however, no significant change of PC by LDIR has been detected (Supplementary Figure 1), indicating that the LDIR-induced alteration of GPAM expression was not apparently associated with PC metabolism. Neither significant change was observed in phosphatidylinositol, phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylglycerol (PG) (data not shown).

Discussion

The aim of this study was to understand how molecular systems of EBV infected-B cells respond to LDIR as a potential determinant influencing the carcinogenesis process. Our iTRAQ quantitative MS approach identified multiple molecular pathways affected in a time-dependent fashion by LDIR in pre-malignant EBV-B cultured under various conditions including those potentially associated with the lymph node microenvironment.

The iTRAQ data were partially consistent with those of the cDNA analysis. For example, iTRAQ detected the signaling activation responsible for the damaged DNA in mono-cultured EBV-B, which was consistent with the findings of cDNA array and RT-PCR and downregulation of amphiregulin (AREG), formin 1 (FMN1), and B-cell lymphoma 2 (BCL2). The bystander effects on EBV-B triggered by the LDIR-exposed MSC included stimulation of ribosomal biogenesis, and ATP synthase, and cell adhesion and migration signaling. Notably, phosphorylation

![Figure 5. LDIR-modulated miRNA alteration. EBV-B cells cultured for 24 h after acute exposure to indicated doses of irradiation without (moB-IR, upper panel) or with MSC condition (coB-IR, lower panel). Expression levels of let-7a, miR-16, miR-19b and miR-21 (A) and miR-27b (B) were evaluated by qRT-PCR. Error bars indicate the standard error of the mean for n = 3 independent experiments. *p < 0.05; **p < 0.01.](image-url)
of cell-surface glycoprotein CD44 was upregulated not only in co-irradiated, but also in bystander, EBV-B cells. These results indicate that LDIR stimulated cell-cell interactions, adhesion, and migration of the EBV-B cells in their microenvironment.

After LDIR exposure, the communication of bioactive substances from irradiated to sham-irradiated bystander cells is known to modify biological responses, and the bystander response can become saturated at relatively low doses irradiation (Prise and O’Sullivan 2009). This demonstrates that the bystander responses of LDIR were essentially as effective as those associated with direct irradiation (Prise et al. 1998, Prise and O’Sullivan 2009; Du and Barcellos-Hoff 2013), which is consistent with our results. This implies that low-dose exposures have considerable effects, including the promotion of secondary cancers, for example, after external beam therapy (Belyakov et al. 2001).

Although two post-radiation time points (i.e., 1 and 24 h) investigated by iTRAQ proteomics detected little overlapping responses, the pathway analysis demonstrated that the connections between the time-points depend on the respective culture conditions. In monocultured EBV-B cells, acute LDIR induced activation of protein folding and proteasomal pathways, which was followed by metabolic pathways upregulation, indicating change over time of the LDIR-induced stress response. In monocultured MSC, LDIR upregulated keratin expression with cell adhesion pathways activation at both post-radiation time points, suggesting the activation of ATP-dependent cytoskeleton and motility-related signaling. Immune response-related signaling in MSC co-cultured EBV-B cells, and cytoskeleton and cell cycle pathways in EBV-B cells under bystander effects of irradiated MSC were commonly activated at the 1- and 24-h time-points.

We observed discrepancies in the detection of GPAM, iTRAQ proteomics could not identify GPAM, although its modulation by LDIR was consistently observed by cDNA array, qRT-PCR, and western blot analysis. These discrepancies might be caused by the lower sensitivity of iTRAQ proteomics compared to western blotting, as well as cDNA array and qRT-PCR. Although iTRAQ detects unknown proteins that can be identified from the protein sequence databases, the sensitivity of protein identification is typically less than typical western blotting, which uses a known antibody. cDNA array and qRT-PCR using specific primers are also highly sensitive for gene identification.

Ionomizing radiation is known to change levels of specific miRNA, and these changes vary by cell type. In this study, we observed that LDIR induced changes in expression of miRNA, let-7a, miR-16, miR-19b and miR-21, as well as a lipid metabolic miRNA hub miR-23b, and that these changes differed by cell type and were accompanied by coordinate modulation of their common target, the lipid biosynthesis enzyme GPAM expression, which were specific to LDIR. To the best of our knowledge, this is the first description of this effect. Our phospholipid analysis showed that LDIR induced GPAM changes in EBV-B cells has no significant relevance with phospholipid content, which is consistent with the findings of the null mouse for GPAT1 (mouse orthologous of GPAM) (Hammond et al. 2002). In turn, GO analysis showed the parallel activation of fatty acid oxidation and GPAM induction by LDIR in EBV-B cells co-cultured with MSC. Since GPAM is known to link with triacylglycerol synthesis (Wendel et al. 2013), the LDIR-induced lipid metabolism including triacylglycerol synthesis is remained to be clarified.

We conclude that LDIR activates cell signaling pathways in EBV-B cells directly, and indirectly via interactions with irradiated neighboring stromal cells, and that miRNA is, at least in part, involved in cell response to LDIR. The data obtained using state-of-the-art techniques, iTRAQ proteomics, present the preliminary screen of marginal changes in protein expression in EBV-B cells and stromal cells by identifying multiple LDIR-sensitive proteins with both known and previously unreported roles in radiation responses, and will serve as a useful database of molecular pathways affected by LDIR.

Acknowledgements

The authors wish to thank Drs Hideki Hayashi and Masayuki Tanaka, Support Center for Medical Research and Education, Tokai University, Kaori Saith, Tomomi Ikekda and Akemi Kawasaki for technical assistance. We thank the Laboratories of Molecular and Biochemical Research and Cell Biology, Research Support Center, Juntendo University Graduate School of Medicine for use of their facilities. We thank Kathryn Hale for manuscript review and Numsen Hall Jr for help in the preparation of the manuscript. This work was supported in part by the Grant-in-Aid for Scientific Research (C), Japan and by Grant-in-Aid (S1311011) from the Foundation of Strategic Research Projects in Private Universities from the MEXT, Japan (to Y.T.).

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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