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Dynamics of the transcriptome response of cultured human embryonic stem cells to ionizing radiation exposure

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ABSTRACT

One of the key consequences of exposure of human cells to genotoxic agents is the activation of DNA damage responses (DDR). While the mechanisms underpinning DDR in fully differentiated somatic human cells have been studied extensively, molecular signaling events and pathways involved in DDR in pluripotent human embryonic stem cells (hESC) remain largely unexplored. We studied changes in the human genome-wide transcriptome of H9 hESC line following exposures to 1 Gy of gamma-radiation at 2 h and 16 h post-irradiation. Quantitative real-time PCR was performed to verify the expression data for a subset of genes. In parallel, the cell growth, DDR kinetics, and expression of pluripotency markers in irradiated hESC were monitored. The changes in gene expression in hESC after exposure to ionizing radiation (IR) are substantially different from those observed in somatic human cell lines. Gene expression patterns at 2 h post-IR showed almost an exclusively p53-dependent, predominantly pro-apoptotic, signature with a total of only 30 up-regulated genes. In contrast, the gene expression patterns at 16 h post-IR showed 354 differentially expressed genes, mostly involved in pro-survival pathways, such as increased expression of metallothioneins, ubiquitin cycle, and general metabolism signaling. Cell growth data paralleled trends in gene expression changes. DDR in hESC followed the kinetics reported for human somatic differentiated cells. The expression of pluripotency markers characteristic of undifferentiated hESC was not affected by exposure to IR during the time course of our analysis. Our data on dynamics of transcriptome response of irradiated hESCs may provide a valuable tool to screen for markers of IR exposure of human cells in their most naive state; thus unmasking the key elements of DDR; at the same time, avoiding the complexity of interpreting distinct cell type-dependent genotoxic stress responses of terminally differentiated cells.

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1. Introduction

The genetic material of every living being is constantly challenged by environmental agents such as background IR and endogenous threats arising as a by-product of normal metabolism. For example, every cell in the human organism on average receives tens of thousands of DNA lesions per day [1]. To counter these deleterious effects produced by genotoxic agents, several cellular mechanisms have evolved to detect the various types of DNA lesions, to signal their presence, and to mediate their repair and removal; all collectively known as DDR. The biological significance of these mechanisms can hardly be overestimated since the accumulation of DNA damage has been thought to play a critical role in the cancerogenesis and the aging processes [2]. Therefore, a great deal of effort has been invested in elucidating the details of molecular signaling and cellular events associated with DDR.

Early work based on using conventional biochemical and genetics techniques identified many components of DDR in human cells [3,4]. One of the key aspects of DDR is the widespread changes in the level of expression of many genes involved in various pathways of cellular metabolism. With the development of newer, high-throughput technology it became possible to interrogate the expression of thousands of genes simultaneously following specific treatment conditions [5], including genotoxic agent exposures [6]. These functional genomics experiments vastly extended our knowledge how human cells of different origins respond to IR [7–11]. DNA microarray profiling enabled identification of novel radiation-responsive signaling pathways, further advancing our understanding of IR effects on humans [12]. However, practically all these experiments were performed on terminally differentiated

Abbreviations: hESC, human embryonic stem cell; IR, ionizing radiation; DDR, DNA damage response; PBS, phosphate buffer saline; BSA, bovine serum albumin; PI, propidium iodide; SAM, Significance Analysis of Microarrays; FDR, false discovery rate; GO, Gene Ontology; DNA DSB, DNA double strand break; IRIF, ionizing radiation-induced foci; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean.

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cells either grown in tissue culture or derived from tissue biopsy specimens. Little is known about how genotoxic stress, particularly IR exposure, affects the global signaling events in pluripotent hESC. The available data primarily focus only on specific genes and distinct pathways involved in IR response of hESC [13–15]. It is known that the human fetus is very susceptible to genotoxic insults [16,17]; but the underlying molecular mechanisms are not fully understood. In addition, hESC are currently under intense research in a context of toxicological studies in which hESC are being employed as a model for therapeutic drug screening [18]. Therefore, identification of novel molecular targets in genotoxic agent screening in pluripotent human cells could significantly contribute to their future use not only in regenerative-based cell replacement clinical strategies, but also for establishing optimized treatment schemes in a personalized medicine [19]. Only one report [20] aimed to define the effects of IR on global changes in gene expression in hESC. However, this study interrogated hESC response only at a single, late timepoint after IR exposures. In the present study, we set out to characterize the dynamics of the transcriptional response of cultured irradiated hESC, to determine the key components of DDR in hESC, and to identify signaling pathways possibly responsible for the sensitivity of early human developmental stage cells to genotoxic IR exposures. To this end, we used a whole human genome-wide functional genomics approach. We show that following 1 Gy of gamma-radiation exposure of H9 hESC "early" DDR is almost exclusively p53-dependent. In contrast, the "late" gene expression signature of irradiated hESC is characterized by robust involvement of a wide range of signaling pathways, many of which seem to be responsible for the survival of hESC.

2. Materials and methods

2.1. Cell culture

Cultured hESCs (H9 cell line, WiCell, Madison, WI, passage 35–40) were routinely grown in mTeSR-1 medium (Stemcell Technologies, Vancouver, Canada) on a BD Matrigel hESC-qualified matrix (BD Biosciences, San Jose, CA) at 37 °C and 5% CO_2. Cell cultures were maintained and expanded following supplier's protocol. Cells were passaged every 5–7 days using collagenase IV (Invitrogen, Carlsbad, CA). The medium was changed every day.

Cell cultures were divided into two groups, and were either exposed to 1 Gy of X-ray irradiation using X-RAD 320 Biological Irradiator unit (Precision X-Ray, Inc., North Branford, CT; dose rate about 1 Gy/min; 320 kV, 12.5 mA); or, alternatively, were mock-irradiated. Cells then were allowed to recover in CO_2 incubator and collected at 2 h and 16 h post-irradiation for analysis.

2.2. Cell proliferation assay

The cells were seeded on Matrigel in equal aliquots before the start of experiment. After IR exposures (0.2 Gy or 1 Gy) and time of incubation post-IR (17 h, 41 h and 65 h), cell cultures were rinsed with phosphate buffer saline (PBS; Invitrogen) to remove detached cells. These floating cells were collected by gentle centrifugation for subsequent cell counting. The attached cells, that are presumably alive, were collected by treatment with Trypsin–EDTA (Invitrogen) for 3 min at 37 °C, and washed with PBS buffer supplemented with 0.5% BSA. Cell count was performed using Z1 Coulter Cell Counter system (Beckman Coulter, Inc., Brea, CA) at least in triplicate for each experimental data point.

2.3. Immunocytochemistry

The cell cultures were grown on glass-bottom LabTek Chamber Slide (BD Biosciences) as described above. Cells were fixed with 4% paraformaldehyde for 10 min, and then permeabilized with 0.1% Triton-X-100 for 5 min. Primary antibodies dissolved in blocking solution containing 3% bovine serum albumin (BSA; Sigma, St. Louis, MO) were added for 1 h, and then appropriately coupled Alexa Fluor secondary antibodies (Invitrogen) were used for indirect immunofluorescent detection of primary antigens, as described in [21]. All secondary antibodies were tested for nonspecific immunoreactivity. The following primary antibodies were chosen: Oct-4, SSEA4, TRA-1-81 and 53BP1 (Santa Cruz Biotechnology, Santa Cruz, CA). DAPI stain was used to identify the nuclei. The antifade media (VectaShield, Vector Laboratories, Inc., Burlingame, CA) was used to mount the coverslips; the samples were examined by Axioplan Zeiss epifluorescent microscope (Carl Zeiss, Thornwood, NY). The microscope and CCD camera image acquisition settings were the same for all corresponding samples. 53BP1 foci assay was performed as described in [22], and at least 200 cells were examined per each datapoint. Foci scoring by eye were typically performed in a blinded manner.

2.4. Cell cycle analysis

Cell cycle analysis was performed using propidium iodide (PI)/Triton-X-100 staining solution (0.1 mg/ml PI; 0.1 mg/ml RNAse A; 0.1% (v/v) Triton-X-100, Sigma). Human ESCs were harvested by collagenase IV treatment and counted with hemocytometer. In total, 500,000 cells were fixed after incubation with 70% ethanol at 4 °C for 2 h and stained in PI/Triton-X-100 staining solution for 30 min at room temperature in dark. The samples were analysed by flow cytometry (FACS Calibur, BD Biosciences) measuring FL2 area versus total counts and with ModFit 3.0 (Verity Software House) to generate percentages of cells in G1, S and G2/M phases

2.5. RNA sample preparation, probe labeling and DNA microarray procedure

The extraction of total RNA was performed using Trizol (Invitrogen), and then RNA preparations were purified with RNeasy kit (Qiagen, Valencia, CA) and TURBO DNA-free kit (Ambion, Inc., Austin, TX) per manufacturers' instructions. The amount and quality of RNA samples were assessed on the Agilent 2100 Bioanalyzer with RNA 6000 Nano Reagents and Supplies (Agilent, Santa Clara, CA) [23]. Agilent RNA Spike-In Mix was added to the RNA samples prior to the labeling reactions following the RNA Spike-In Kit protocol. Subsequently, cRNA targets were synthesized from 1 μg of total RNA in each reaction and fluorescently labeled with Cy5-CTP (PerkinElmer, Waltham, MA) in separate labeling reactions using the Agilent Quick-Amp Labeling kit. The Universal Reference RNA (Stratagene, La Jolla, CA) was used to synthesize Cy-3-CTP (PerkinElmer, Waltham, MA) labeled cRNA sample to include as a common reference target throughout all experiments. The dual-labeled cRNA targets corresponding both to experimental and reference samples were combined and hybridized to 4x44k Agilent Human Whole Genome oligo microarrays using Agilent SureHyb hybridization chambers. Protocols for microarray hybridization and washing were as suggested by manufacturer. Hybridized DNA microarrays were scanned with a resolution of 5 μm on an Agilent DNA microarray scanner enabled by SureScan High-Resolution Technology (Agilent), and TIFF images were subsequently processed by Feature Extraction 10.5 software (Agilent). All samples had four independent biological replicates, and each replicate was run on a separate array.

2.6. Data analysis

Raw data derived from processing TIFF image files were analysed using BRB-Array Tools Version 3.8.0 software developed by Dr. Richard Simon and Amy Peng Lam (Biometric Research Branch, National Cancer Institute, NIH). Time-matched irradiated versus mock-irradiated samples were used for analysis to determine the radiation–responsive genes from quadruplicate independent experiments per each data point. Differentially expressed genes were identified using a Significance Analysis of Microarrays (SAM) method [24]. We identified genes that were differentially expressed among the two classes (irradiated hESC versus mock-irradiated hESC cultures) by using a multivariate permutation test [25]. We used the multivariate permutation test to provide a median false discovery rate (FDR) of 10%. The FDR is the proportion of the list of genes claimed to be differentially expressed that are false positives. The test statistics used are random variance *t*-statistics for each gene [26]. The sets of differentially expressed genes were tested for functional significance using the DAVID Bioinformatics Resources 2008 [27]. This tool obtains the Gene Ontology (GO) annotations from a database and generates a statistical analysis of the functional annotations that are overrepresented in the selected set of genes. A Bonferroni correction for multiple comparisons was included in the analysis [28]. GO biological processes with EASE scores less than 0.05 were considered to be statistically significant [29]. Minimum Information About a Microarray Experiment (MIAME)-compliant raw data for our experiments have been uploaded onto the ArrayExpress database maintained by the European Bioinformatics Institute (accession no. E-MEXP-2596).

2.7. Quantitative real-time PCR

The quantitative RT-PCR was done on RNA samples from three independent cell culture experiments. The complementary DNA was synthesized from total RNA using One-Step RNA-to-C_t kit (Applied Biosystems) according to the manufacturer's protocol. For each gene, PCR reactions were run in triplicate on one sample. RT-PCR was performed on iCycler iQ (Bio-Rad, Inc.) in 20-μl reactions by using TaqMan Assay-on-Demand primers/probe sets (Applied Biosystems) for the following genes: *CDKN1A*, *GADD45A*, *DHFR*, *CCDC88A* and *MT1F*. Quantitative RT-PCR data were analysed as in [30].