Gene 455 (2010) 8–15

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Expression of pluripotency-associated genes in the surviving fraction of cultured human embryonic stem cells is not significantly affected by ionizing radiation

Mykyta V. Sokolov, Irina V. Panyutin, Mykola I. Onyshchenko, Igor G. Panyutin *, Ronald D. Neumann

Radiology and Imaging Sciences, Clinical Center, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892, USA

ARTICLE INFO

Article history:
Received 24 November 2009
Received in revised form 8 January 2010
Accepted 24 January 2010
Available online 1 February 2010

Received by S.M. Mirkin

Keywords:
Human embryonic stem cells
Genotoxic agent
Pluripotency marker
Ionizing radiation
Directed differentiation

ABSTRACT

Human embryonic stem cells (hESC) are capable to give rise to all cell types in the human body during the normal course of development. Therefore, these cells hold a great promise in regenerative cell replacement based therapeutical approaches. However, some controversy exists in literature concerning the ultimate fate of hESC after exposure to genotoxic agents, in particular, regarding the effect of DNA damaging insults on pluripotency of hESC. To comprehensively address this issue, we performed an analysis of the expression of marker genes, associated with pluripotent state of hESC, such as Oct-4, Nanog, Sox-2, SSEA-4, TERT, TRA-1-60 and TRA-1-81 up to 65 h after exposure to ionizing radiation (IR) using flow cytometry, immunocytochemistry and quantitative real-time polymerase chain reaction techniques. We show that irradiation with relatively low doses of gamma-radiation (0.2 Gy and 1 Gy) does not lead to loss of expression of the pluripotency-associated markers in the surviving hESC. While changes in the levels of expression of some of the pluripotency markers were observed at different time points after IR exposure, these alterations were not persistent, and, in most cases, the expression of the pluripotency-associated markers remained significantly higher than that observed in fully differentiated human fibroblasts, and in hESCs differentiated into definitive endodermal lineage. Our data suggest that exposure of hESC to relatively low doses of IR as a model genotoxic agent does not significantly affect pluripotency of the surviving fraction of hESC.

Published by Elsevier B.V.

1. Introduction

Human embryonic stem cells (hESCs) possess the capacity to differentiate into all cell types in the body (pluripotency) and, as such, can serve as a valuable model of embryonic development. Human ESCs are an ultimate source of differentiated cells that may be used in cell-based substitutive therapy (Liew et al., 2005). To fully benefit from the regenerative potential of hESCs in clinical settings one has to anticipate problems inherent to the unique biological characteristics of ES cells. The key properties of ES cells under normal conditions are their ability to self-renew and to maintain pluripotency. However, published data concerning the ultimate fate of ES cells after exposure to genotoxic stress are somewhat contradictory. On the one hand, both murine, non-human primate and human ES cells were shown to be hypersensitive to DNA damaging agents and respond by undergoing apoptosis and/or differentiation (Aladjem et al., 1998; Hong and Stambrook, 2004; Lin et al., 2005; Qin et al., 2007). It is also known that the developing human embryo is considered to be among the most vulnerable to genotoxic agent exposures (McCollough et al., 2007). On the other hand, a more recent study suggests that hESC maintain pluripotency for at least 24 h after 2 Gy of IR exposure (Momcilovic et al., 2009). Hence, how DNA damaging agents, for instance, IR exposure with relatively low doses, might affect the pluripotency state of hESCs remains to be addressed.

The key regulators of pluripotency are transcription factors Oct-4, Nanog and Sox-2; they are found to be expressed in undifferentiated stem cells (Matin et al., 2004; Boyer et al., 2005; Hyslop et al., 2005). Together with these factors comprising the core of the transcription regulatory circuitry underlying undifferentiated state of stem cells, hESCs can be characterized by the expression of SSEA-4, TRA-1-60, TRA-1-81 and TERT (Ginis et al., 2004; Fong et al., 2009). In order to shed light on how genotoxic stress such as IR affects the pluripotent state of hESC in culture, in this study we comprehensively characterized the expression of these markers after IR exposures of hESC using three independent methodologies. In addition, in this study we cultivated hESC using feeder free conditions to avoid potential effects of MEFs on the measurements of expression of pluripotency markers.

Abbreviations: hESC, human embryonic stem cells; ES cells, embryonic stem cells; IR, ionizing radiation; qRT-PCR, quantitative real-time polymerase chain reaction; EMEM, Earle's modified Eagle's medium; DMEM, Dulbecco's modified Eagle's medium; MEF, murine embryonic fibroblasts; KSR, Knockout serum replacement; bFGF, basic fibroblast growth factor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PI, propidium iodide.

* Corresponding author. 9000 Rockville Pike, Bldg 10, Room 1C492, Bethesda, MD 20892, USA. Fax: +1 301 480 9712.

E-mail addresses: sokolovm@mail.nih.gov (M.V. Sokolov), ipanyutinv@mail.nih.gov (I.V. Panyutin), onyshchenkom@mail.nih.gov (M.I. Onyshchenko), ipanyuting@mail.nih.gov (I.G. Panyutin), rneumann@mail.nih.gov (R.D. Neumann).

0378-1119/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.gene.2010.01.006

2. Materials and methods

2.1. Cell lines and cell culture

Initially hESCs (H9 cell line, WiCell, Madison, WI, passage 35–40) were maintained on a feeder layer of irradiated MEFs using medium consisting of 80% Knockout Dulbecco's modified Eagle's medium (KO-DMEM, Invitrogen, Carlsbad, CA) supplemented with 15% Fetal bovine serum (Invitrogen), 5% Knockout serum replacement (KSR, Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO), 1% non-essential amino acids, 2 mM L-Alanyl-L-glutamine and 4 ng/ml basic fibroblast growth factor (bFGF, Invitrogen). Cell cultures were passaged using Collagenase IV (Invitrogen) every 6–7 days, only phenotypically uniform hESC colonies were collected. Subsequently, hESCs were transferred to feeder-independent culture conditions, using BD Matrigel hESC-qualified Matrix (BD Biosciences, San Jose, CA), and grown in mTeSR-1 (Stemcell Technologies, Vancouver, Canada) at 37 °C and 5% CO_2. Cell cultures were maintained and expanded following the manufacturer's protocol. The medium was changed every day.

BJ and IMR-90 normal human diploid fibroblasts (ATCC, Manassas, VA) were grown in Earle's modified Eagle's medium (EMEM) supplemented with 10% fetal bovine serum, non-essential amino acids, 1 mM sodium pyruvate and 2 mM L-glutamine (Invitrogen) at 37 °C and 5% CO_2 and passaged every 5–7 days using 0.5% Trypsin-EDTA.

Exposure of cells to ionizing radiation was accomplished as follows: cultured cells were divided into three groups and were exposed either to 0.2 Gy or 1 Gy of ^{60}Co gamma-radiation using Eldorado 8 ^{60}Co teletherapy unit (MDS Nordion, Ottawa, Ontario, Canada, formerly Atomic Energy of Canada, Ltd.; dose rate about 1 Gy/min), or, alternatively, were sham-irradiated. Cells then were returned to CO_2 incubator and collected at 17 h, 41 h and 65 h post-irradiation for analysis. These time points correspond to approximately 1, 2 and 3 average duplication time for H9 hESC line (Becker et al., 2006).

2.2. Directed differentiation of hESC into definitive endoderm

H9 hESCs were seeded onto 6-well plates covered with BD Matrigel hESC-qualified Matrix (BD Biosciences) at 10^5 cells per well. Then, the cells were maintained in mTeSR1 medium at 5% CO_2 and 37 °C for two days with the medium changed every day. Starting from day three cells in culture were maintained in differentiation medium (DMEM/F12 supplemented with 20% KSR, 100 ng/ml Activin A, 4 ng/ml bFGF and 20 μM LY294002), which was changed every day (McLean et al., 2007). After four days of differentiation cells were trypsinized and collected for further studies.

2.3. Immunocytochemistry

For immunohistochemistry cells were grown on glass-bottom LabTek® two-well Chamber Slide™ (BD Biosciences) in the feeder-free conditions described above. The cell cultures were fixed with 4% paraformaldehyde for 10 min, and then permeabilized with 0.1% Triton-X-100 in phosphate-buffered saline (PBS) for 5 min. Primary antibodies were applied for 1–2 h (overnight at 4 °C for cleaved caspase 3), and appropriately coupled Alexa Fluor secondary antibodies (Invitrogen) were used for single or double labeling for 1 h. All secondary antibodies were tested for nonspecific immunoreactivity. The following primary antibodies were used: Oct-4, SSEA4, TRA-1-81, Nestin, and Sox7 (Santa Cruz Biotechnology, Santa Cruz, CA), cleaved caspase 3 (Cell Signaling Technology, Danvers, MA), TERT and Brachyury (Abcam, Cambridge, MA). DAPI stain was used to identify the nuclei. After mounting in antifade media (VectaShield, Vector Laboratories, Inc., Burlingame, CA), the samples were examined by Axioplan Zeiss epifluorescent microscope (Carl Zeiss, Thornwood, NY). The camera exposure time and microscope settings were kept constant across all corresponding samples.

2.4. Cell viability and flow cytometry

At the indicated time points, flasks containing hESCs were rinsed with PBS supplemented with 0.5% bovine serum albumin (BSA, Sigma) to remove detached cells. Then the remaining cells, that we call surviving fraction, were collected by treatment with Trypsin-EDTA for 3 min at 37 °C, and washed three times with PBS buffer supplemented with 0.5% BSA. Before the third wash cell pellet was resuspended in 1 ml of the same buffer, and 50 μl aliquot was taken into Trypan Blue exclusion assay. Cell count was performed using hemacytometer for each aliquot immediately after addition of equal volume of Trypan Blue.

To assess the viability of hESC in colonies after IR exposures, cells were incubated at 37 °C for 1 h with Hoechst 33342 (8 μg/ml; Molecular Probes, Eugene, OR) and propidium iodide (PI, 20 μg/ml; Sigma, St. Louis, MO). Hoechst 33342 is known to stain the nuclei of both live and dying cells whereas PI penetrates the cell membrane of only dying/dead cells. Cell colonies were visualized using an inverted fluorescence microscope (Axiovert 200 M, Thornwood, NY) equipped with a fluorescent light source.

For flow cytometry experiments, each sample was diluted with PBS/0.5% BSA buffer yielding a total of 3×10^5 cells. For analysis of the expression level of cell surface antigens, cells were incubated either with SSEA-4-phycoerithrin (PE)-conjugated antibody (R&D Systems, Minneapolis, MN), or with TRA-1-60 antibody (Santa Cruz Biotechnology) and, after two washes with PBS/0.5% BSA buffer, with secondary FITC-conjugated antibody (Vector Laboratories, Inc.). Prior to staining, cells were blocked with human IgG for 15 min at room temperature. For intracellular staining with Oct-4-PE and Sox2-PE conjugated antibody (R&D Systems), the cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed two times with PBS and permeabilized with 0.1% saponin (Sigma) in PBS prior to incubation with antibodies. All antibody incubations were performed according to the manufacturer's instructions. Isotype controls were included for each antibody staining.

After staining, cells were washed and resuspended in PBS. Fluorescence activated cell sorting (FACS) analysis was performed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) utilizing an emission wavelength of 488 nm and a 525 nm excitation detector. Cell Quest Pro software was used for both data acquisition and analysis to produce histogram plots and median peak values. As a control for nonspecific binding for each conjugated antibody we used the same IgG subclass with the same fluorochrome conjugation and for non-conjugated antibody — the same IgG subclass conjugated to fluorochrome. A total of 10,000 events were acquired for each analysis.

2.5. Quantitative RT-PCR

Cells were trypsinized, washed two times with PBS and finally suspended in PBS with a concentration of 10,000 cells per μl. cDNA was synthesized using SuperScript III CellsDirect cDNA System (Invitrogen) according to the manufacturer's recommendations. Quantitative real-time PCR (qPCR) was performed on iCycler iQ instrument (Bio-Rad, Hercules, CA) using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen). Primers were purchased from Qiagen (Quantitech Primer Assays, Valencia, CA). PCR protocol consisted of 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles (95 °C — 15 s, 55 °C — 30 s, 72 °C — 30 s) according to Quantitech Primer Assay recommendations. Ct (cycle threshold) values were obtained for each sample, averaged over triplicates in two biological replicates and normalized to beta-actin, according to the formula $E = 2^{(\mathrm{Ct[beta\text{-}actin]} - \mathrm{Ct[studied\ gene]})}$,