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MINIREVIEWS

# Changes in human pluripotent stem cell gene expression after genotoxic stress exposures

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## Abstract

Human pluripotent stem cells (hPSCs) represent heterogeneous populations, including induced pluripotent stem cells (iPSCs), endogenous plastic somatic cells, and embryonic stem cells (ESCs). Human ESCs are derived from the inner cell mass of the blastocyst, and they are characterized by the abilities to self-renew indefinitely, and to give rise to all cell types of embryonic lineage (pluripotency) under the guidance of the appropriate chemical, mechanical and environmental cues. The combination of these critical features is unique to hESCs, and set them apart from other human cells. The expectations are high to utilize hESCs for treating injuries and degenerative diseases; for modeling of complex illnesses and development; for screening and testing of pharmacological products; and for examining toxicity, mutagenicity, teratogenicity, and potential carcinogenic effects of a variety of environmental factors, including ionizing radiation (IR). Exposures to genotoxic stresses, such as background IR, are unavoidable; moreover, IR is widely used in diagnostic and therapeutic procedures in medicine on a routine basis. One of the key outcomes of cell exposures to IR is the change in gene expression, which may underlie the ultimate hESCs fate after such a stress. However, gaps in our knowledge about basic biology of hESCs impose a serious limitation to fully realize the potential of hESCs in practice. The purpose of this review is to examine the available evidence of alterations in gene expression in human pluripotent stem cells after genotoxic stress, and to discuss strategies for future research in this important area.



**Key words:** Human pluripotent stem cells; Gene expression alterations; Genotoxic stress; Ionizing radiation

**Core tip:** Genome-wide alterations in gene expression in human pluripotent stem cells (hPSCs) following genotoxic stress exposures may underlie the ultimate fate and outcome of practical utility of hPSCs which makes systematic studies of these effects a high priority in stem cell research.

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## INTRODUCTION

Human pluripotent stem cells have been isolated from the inner cell mass of the preimplantation embryos (embryonic stem cells, hESCs)[1], from fetal germ cells (embryonic germ cells, hEGCs)[2], and, more recently, from breast tissues of healthy human subjects (endogenous plastic somatic cells, ePSCs)[3]. Since the discovery of so-

called Yamanaka factors in 2006[4], a variety of different types of adult human somatic cells were experimentally converted into so-called induced pluripotent stem cells (iPSCs) in many respects resembling hESCs. Recent advances in application of somatic cell nuclear transfer technology (SCNT) to human cells led to breakthroughs in producing human pluripotent stem cells almost indistinguishable from hESCs[5,6]. Arguably, the most studied among different types of human pluripotent stem cells are hESCs. These cells readily demonstrate a stable developmental potential to form derivatives of all three embryonic germ layers, and can be kept in the undifferentiated state in culture for prolonged periods, if not indefinitely. Human pluripotent stem cells are promising candidates for development of novel models to study human developmental biology, to promote drug discovery, and to foster efforts for cell-based regenerative medicine. To realize the potential of hESCs in practice would require growing and expansion of these cells in culture, during which hESCs may face many challenges. For example, hESCs experience culture stress, and stress associated with genotoxic agents, ubiquitous in nature.

In real life situations, exposures to electromagnetic ionizing radiation (IR) stemming from cosmic rays, natural background radioactive isotopes, and many other sources are inevitable. Many studies indicate IR as being one of the most potent cytotoxic and genotoxic agents[7,8]. One of the key manifestations of the biological effects of IR is the change in global gene expression, which may dictate the ultimate hESCs fate after genotoxic stress. Detailed analyses of the available evidence of alterations in gene expression in human pluripotent stem cells after IR exposures will help pave the way for future research and strategical planning in this important area of studies.

## GENE EXPRESSION-SPECIFIC SIGNATURE OF HESCS

The global gene expression signature of hESCs has been examined by many modern assays, including serial analysis of gene expression (SAGE), DNA microarray analysis, and new-generation, massively parallel signature sequencing (NGS). As a result of these studies, some key genes that regulate pluripotency and self-renewal, were identified and verified as being expressed in all lines of undifferentiated hESCs, such as *POU5F1*, *SOX2*, *NANOG*, and several others[9-11]. A remarkable heterogeneity and variability in gene expression was found in many functional classes of genes across multiple lines of hESCs, including but not limited to housekeeping genes, and some "stemness" genes, such as *STAT3* and *RUNX1*[12]. A high degree of both quantitative and qualitative differences in gene expression among hESC lines exist for many genes; and some of these differences may contribute to measurable biological consequences. For example, different developmental outcomes may result from a relatively moderate, *i.e.*, less than 2-fold variation, in the level of expression of *POU5F1* in hESCs. *REM2* is up-regulated in hESCs and is necessary to maintain survival and pluripotency of hESCs by down-regulating p53 and cyclin D1[13]. Human ES cells are distinct from somatic cells in the expression of members of the E2F family and RB family so-called pocket proteins, such as p105 (RB1), p107 (RBL1), and p130 (RB2) that are known to control expression of genes implicated in both DNA and nucleotide metabolism[14]. Some other distinct subsets of genes are expressed at consistently higher levels in hESCs compared to normal differentiated human cells. Among these are both components of telomerase *TERT* and *TR*[15], antioxidant genes, such as *SOD2* and *GPX2*[15], and many DNA repair genes, such as *BRCA1*, *MSH3*, *MSH6*, *LIG3*, *DMC1*, *FEN1*, *RPA3*, *BLM*, *WRN*, *etc.*[15,16], partly explaining higher fidelity of DNA repair in hESC after genotoxic stress exposures[17,18]. Importantly, some genes encoding key proteins implicated in cell cycle control and DNA damage signaling were also observed to be more abundantly expressed in hESCs compared to IMR-90 fibroblasts. Among them are *ATR*, *CHEK1*, *PCNA*, *PRKDC* (DNA-PKcs), and others[19]. Recently, it was demonstrated that levels of BCL-2 are lower , whereas those of pro-apoptotic PUMA are higher, in hESCs compared to human somatic cells[20], which is in concert with the tendency of hESC to undergo programmed cell death under permissive conditions. Noteworthy, the hybrid sequencing technique identified that a substantial subset of 273 novel RNAs from gene loci is expressed in human pluripotent stem cells, but not in diverse fetal and adult tissues, further adding to the differences in gene expression signatures between human pluripotent stem cells and other types of cells[21]. The unique epigenetic landscape of the former might contribute, at least in part, to those distinct transcription profiles observed in many studies[22,23].

## CHANGES IN PROTEIN-CODING GENE EXPRESSION IN IRRADIATED HESCS

The transcriptional responses of many types of fully differentiated somatic human cells exposed to IR have been studied by numerous labs in the past. Much less is known about how human pluripotent stem cells, such as hESCs, respond to genotoxic stresses at the level of whole genome gene expression. Studies into such gene expression alterations were conducted only recently; but, we still have only partial knowledge about hESCs transcriptional programs elicited by DNA damage/genotoxic stressors. Importantly, changes affecting the global gene expression networks have been strongly associated with ultimate cell fates/outcomes in human cells undergoing genotoxic stress exposures. Such perturbations are considered to be an integral part of human cell response to DNA damage-induced stress[24,25].

Comprehensive studies specifically aimed at understanding how global gene expression alterations manifest

in human pluripotent stem cells are scarce, and cover very limited number of hESC lines, *i.e.*, the most widely used H9 and H1 hESC lines[23,26,27]. DNA microarray technique was used to analyze the transcriptional changes in H9 cell line of hESCs 24 h after 0.4 Gy, 2 Gy, and 4 Gy of gamma-radiation[26]. Quite unexpectedly, it has been found that the expression levels of a set of core transcription factors governing pluripotency, in particular, and stemness, in general, in hESCs are not changed significantly by IR exposures up to 4 Gy of gamma-radiation[26]. The most common themes involved in manifestation of response of IR-exposed hESCs include p53 stress signaling, cell death/apoptosis, cell cycle regulation, developmental processes, and many others.

The key genes that were initially discovered as being IR-responsive in fully differentiated adult human cells, such as *CDKN1A*, *GADD45A*, *BTG2*, and some others, appear to be upregulated by genotoxic stress exposures in human pluripotent stem cells as well[23,26]. The effect of induced expression of stress response genes is clearly dose-dependent, since low doses of genotoxic stressors may not elicit robust changes in transcriptional responses in hESCs[28]. A modest dose (0.4 Gy) of gamma-radiation was found to have an impact on cell death, cancer, and p53 signaling pathways; IR exposure with this dose apparently failed to significantly reduce hESCs proliferation at 24 h post-IR[26].

Importantly, much higher dose of 2 Gy of gamma-radiation led to changes in canonical TFG-β and Wnt/β-catenin signaling, including *WNT10A* (up 2.1-fold), *WNT9A*, and *TGFBR2*[26]. The perturbations in Wnt signaling axes following IR exposures could potentially affect the ultimate fate of irradiated hESCs, since Wnt genes are involved in key developmental pathways in human pluripotent stem cells[29,30]. This dose induced *CDKN1A* overexpression by 2.3-fold in H9 hESCs[26]. Noteworthy, the expression levels for many genes implicated in general metabolism functions (molecular transport *SLC6A13*, *SLC25A13*, cell morphology, amino acid metabolism, *etc.*) were significantly altered in hESCs by 2 Gy of IR exposures[26].

Despite a high degree of similarity in gene expression profiles observed both after 2 Gy and 4 Gy of IR exposures, p53 and aryl hydrocarbon signaling, cancer-related processes, cell death, cell cycle and proliferation were found to undergo major modulations in hESCs after the higher dose (4 Gy). Among the highly induced IR-responsive genes were key genes implicated in p53 stress signaling, such as *CDKN1A*, *TP53INP1*, *HDM2* and TNF receptor genes[26]. The minor gene expression alterations observed in the differentiation processes failed to lead to a loss of pluripotency even after 4 Gy of IR exposures. Unexpectedly, the expression changes of the core transcriptional factors operating in hESC were quite minor; hence, successful formation of teratomas was proven to be feasible to achieve even after 4 Gy. One of the key conclusions of this study is that the gene expression changes in H9 line of hESCs are dose-dependent at a late timepoint after IR (24 h)[26]. However, it remains to be addressed if this finding is still valid for other time points after IR exposures; and, if it can be generalized to other lines/types of human pluripotent stem cells.

Our more recent work examined the dynamic changes in global gene expression of H9 hESC line after 1 Gy of IR both at 2 and 16 h post-exposures[23]. There were major differences in transcriptome alterations in hESCs and somatic human cell lines, such as fibroblasts, following IR[23,31,32]. Overall, the scale of gene expression changes was rather modest, with a total of only 30 overexpressed genes observed in H9 hESC at an "early" timepoint after 1 Gy exposures. At the earliest, changes in expression cover almost entirely a limited subset of p53 stress signaling pathway genes[23]. For example, the great preponderance of pro-apoptotic/cell cycle arrest gene upregulation in H9 hESC line represent genes, such as *BTG2*, *CDKN1A*, *GADD45A, SESN1*, and *IER5*, that were shown previously to be IR-responsive in human somatic cells[32-34]. Both cell cycle arrest (*GADD45A, PLK2, PLK3, IER5* implicated in execution of G(2)/M checkpoint) and pro-apoptotic genes (*BBC3*, *FAS*, *GDF15*, *HTATIP2*, *CARD8*, *TP53INP1*) were found to be induced by IR exposures at 2 h post 1 Gy of treatment[23]. It is not clear if all these genes are overexpressed in all the cells within irradiated hESC populations, or there are distinct subpopulations of pluripotent stem cells that are destined to follow divergent paths (either recovery after IR-inflicted damage, or cell death). Single-cell methodological approaches may address this important issue in the near future. Detailed studies of gene expression changes at the later (16 h) post 1 Gy of IR identified 354 differentially expressed genes in H9 hESC line[23]. Importantly, the overexpression of many pro-survival genes were observed, for example many members of the metallothionein superfamily, such as *MT1M*, *MT1L*, and *MT1H*[23,32-34], and many genes belonging to general metabolism signaling. Some of the genes that tend to be overexpressed at 16 h post 1 Gy of IR encode known and putative transcription factors, such as *SP5*, *ZNF302*, *ZNF33A*, and *ZFYVE16*. The magnitude of expression of genes that were shown to be upregulated is within 1.5-fold to 25-fold over mock-irradiated hESC cultures[23].

It is noteworthy that the gene expression profiles portraying dynamic transcriptomic changes as part of a broader radioresponse of hESCs cultures to 1 Gy of IR are distinct depending on time after genotoxic stress exposures[23]. Only six genes (*CDKN1A*, *GDF15*, *SESN1*, *BTG2*, *ANKRA2* and *PLK3*) are differentially expressed at both early (2 h) and late (16 h) timepoints examined. This finding could potentially be explained by distinct molecular mechanisms operating in IR-exposed hESC populations at different timepoints after IR. Integration of the gene-rich metadata from other independent "omics" approaches (DNA/histone chemical modifications, non-coding RNAs, *etc.*) would definitely enable researchers to come up with a refined genotoxic stress-induced molecular signature that could be used as a bio-

marker of IR exposure of hESCs.

Recently, the studies in H1 line of hESCs exposed to 1 Gy of IR identified cell growth and proliferation, cell death, DNA-related processes, such as replication, recombination, and DNA repair as being the most genotoxic stress-affected biological pathways/themes[27]. Therefore, it seems that there exists at least partial overlap in major sets of broadly defined processes/functions across distinct hESC lines[23,26,27].

Surprisingly little is known on how low and very low levels of genotoxic stress exposures affect gene expression in hESCs. To the best of our knowledge, our group was the first recently to study the alterations in expression of stress-responsive genes following low and very low doses of IR, such as 0.01 Gy, 0.05 Gy, and 0.1 Gy[28]. The results clearly indicate the heterogeneity of hESCs populations and warrant further genome-wide studies to support the development of "low-dose" specific signature of responses of hESCs.

Pluripotent human stem cells are known to present a high degree of heterogeneity in gene expression, but only recently the possible cause of such diversity was identified by detailed single-cell gene expression studies in hESC subsets defined by surface antigen expression[35]. It was shown that hESC cultures exist as a continuum of intermediate pluripotent cell states[35]. The bulk of the hESC population may express all key pluripotency transcription factors, such as *POU5F1*, *NANOG, SOX2*, *etc.* enabling successful differentiation into derivatives of all three germ layers upon permissive conditions[35,36]. However, a small fraction of hESCs within population shows no lineage priming; these cells possess expression of a particular subset of intercellular signaling molecules with common regulation[35]. Therefore, cultured hESCs can be considered as an inherently quasi-stable population with a multitude of pluripotent states that become committed for lineage specification at some point. The increased expression of developmental regulators in G1 cell cycle might be one of the factors influencing the heterogeneity of hESC populations[37].

The notorious heterogeneity of any stem cell population was recently addressed by single cell quantitative RT-PCR method. It was found that each hESC has high expression in *POU5F1*, but *NANOG* expression levels varies significantly[38]. In addition, geometrical position of individual hESCs within each colony can dictate the preponderance to differentiation along specific developmental pathway, such as ectoderm derivatives from the central part of the colony, trophectoderm from the outer colony ring, *etc.*[39]. This propensity is reflected by notorious differences in basal gene expression among single hESCs within colony[39]. Whether genotoxic stress exposures increase or decrease such heterogeneity in gene expression among distinct hESCs is still unknown. However, the stochasticity of intranuclear molecular reactions and biochemical processes may control the ultimate decision of cell fate associated with DNA damage[40].

## CHANGES IN MICRORNA GENE EXPRESSION IN HESCS EXPOSED TO RADIATION

Gene expression alterations might be heavily influenced by epigenetic changes, such as DNA methylations, histone modifications, and perturbations in miRNA gene expression[31]. It was found that dozens of miRNA genes were overexpressed after UV-exposures in hESCs, including genes belonging to miR-302 and miR-371-372 clusters thought to be human pluripotent stem cell-specific[41]. Importantly, *miR-302a*, *miR-302b*, *miR-302c*, *miR-302d*, and *mir-372* genes were implicated in regulating the expression of p21 in hESCs, governing crucial self-renewal and cell cycle processes[41,42].

The comprehensive data on epigenetic alterations in stressed hESCs are lacking; however, our recent study addressed hESC responses to IR exposures at a level of global microRNAome changes[43]. By employing DNA microarray approach, we showed for the first time, that the microRNAome undergoes global alterations in hESCs after IR. We profiled expression of 1090 miRNA species in irradiated H1 and H9 lines of hESCs, and our analysis revealed statistically significant changes in expression of 54 genes following 1 Gy of IR exposures[43]. Noteworthy, global microRNAome alterations in hESCs were both time-dependent and cell-line-dependent. "Late" transcriptional response at 16 h post-IR exposures of hESCs was shown to be quite robust at a level of global microRNAome. Just a few miRNA genes, such as miR-15b, mir-1973, *etc.*, were IR-responsive at 2 h post IR in both hESC lines we examined. The level of miRNA gene expression alterations at this "early" timepoint was modest at best (usually less than 2-fold)[43]. Our global analysis of microRNAome changes reinforced the idea that miRNA gene expression after genotoxic stress exposures maintains the pluripotent state of surviving hESCs; and, for the most part, implicates the cell cycle-, and alternative splicing-related biological processes. Importantly, the identification of novel molecular targets of genotoxic stress exposure in hESCs will aid in understanding the underlying mechanisms governing the fundamental principles of human pluripotent stem cell behavior and plasticity for application in health science and as a remedy to cure diseases.

## CHANGES IN GENE EXPRESSION IN HESCS EXPOSED TO GENOTOXIC DRUGS

In general, data on sensitivity and gene expression changes in human pluripotent stem cells in response to different genotoxic agents/drugs are still very limited. Studies were performed on comparison of the sensitivities of hESCs, their fibroblast-like derivatives, and matched human iPSCs and their parental and filial fibroblast-like cells

to one of the genotoxic drugs most widely used in clinical practice, such as etoposide which is a known poison of DNA topoisomerase II[44]. It was found that human pluripotent stem cells are exquisitely sensitive to this genotoxic agent compared to differentiated cells, with DNA damage occurring as a result of stem cell exposure to only 0.5 ug/mL concentration of etoposide[44]. Incubation of hESCs with 0.2 umol/L etoposide for 16 h resulted in 80% hESC death[45]. The minor surviving fraction of hESC that recovered after etoposide treatment displayed undifferentiated morphology, even though the ability of these cells to differentiate into derivatives of all three germ layers was not directly examined[45]. The altered expression of key apoptosis regulators such as *TP53* and *BBC3* can at least partly explain a rapid and extensive induction of apoptosis in etoposide-treated hESC cultures[46].

A high degree of sensitivity of hESC cultures to camptothecin, an inhibitor of DNA topoisomerase I, was recently observed[47]. Camptothecin exerts its cytotoxic effects by inducing DNA double-strand breaks (DSBs) in S-phase cells[48]. Even though *CDKN1A* mRNA was induced almost 5-fold compared to sham-treated H9 hESC cultures (1 μmol/L camptothecin, 3 h post exposure), the level of p21 protein remained undetectable[47]. This report also supports the prevailing view that P53 signaling pathway is crucial in execution of apoptosis and in preventing the propagation of DNA damage in genotoxic stress-exposed hESC cultures.

Very recently, the adriamycin-induced DNA damage response in hESCs was characterized with ChIP-seq and microarray analysis[49]. About 1,326 genes were responsive to adriamycin in H9 line of hESCs, with TP53-target genes being implicated mostly in cell death, cell cycle ($P < 10^{-6}$), and cell motility and migration ($P < 10^{-4}$). TP53 was found to target highly distinct subsets of genes during genotoxic stress exposures compared to induced differentiation in hESCs, resulting in specific outcomes that partly overlap, but largely differ[49]. Importantly, genotoxic stress - induced targets of TP53 in hESCs, human colon cancer cells, and human normal cells, such as fibroblasts and keratinocytes, are surprisingly different[50-52]. Therefore, changes in DNA damage-elicited gene expression are governed not only by stimulus-specific upstream signaling, but cellular milieu as well.

Induction of apoptosis was observed as a default response to moderate and high levels of genotoxic stress in hESCs in many studies[20,53-55]. One of the radiomimetic drugs, neocarzinostatin, was shown to elicit a robust programmed cell death at concentrations as low as 0.1 μg/mL in H1 line of hESCs[20]. There were dramatic differences in how pro-apoptotic gene expression alterations manifest; for example, the levels of *BAX* remained unchanged, whereas *BBC3*, *FAS*, *APAF1*, and *NOXA* changed more than 2-fold[20]. High mitochondrial priming of hESCs which is mostly dependent upon the specific characteristics of gene expression in human pluripotent cells may explain, at least in part, hESCs sensitivity to DNA damage - induced apoptosis.

## GENE EXPRESSION ALTERATIONS IN HUMAN INDUCED PLURIPOTENT STEM CELLS EXPOSED TO IONIZING RADIATION

The systematic studies of how human iPSCs (hiPSCs) change their global gene expression in response to genotoxic stresses including IR exposures are yet to be performed. However, previous experiments suggested that the stress gene expression in hiPSCs closely resemble that in hESCs after IR in many respects[19]. Firstly, the expression level of core transcription factors governing pluripotency, such as OCT4 and *NANOG* was not changed significantly in hiPSCs following 1 Gy of IR[19]. Secondly, more than two-fold overexpression of *CDKN1A, GADD45A, PPM1D, SESN1, SESN2,* and *HDM2* genes were observed, suggesting that TP53 signaling is activated after IR exposures in hiPSCs[19]. Thirdly, no changes in the level of total ATM, CHEK2 and NBS1 were detected after genotoxic stress in these cells which was in contrast with the increase in total TP53[19]. In general, observed changes in gene expression, if any, are in concert with alterations in hESC, but the absolute levels of specific alterations may differ[19]. Undoubtedly, future studies using different approaches and protocols to create hiPSCs from different donors and various tissues will strenghten our understanding of transcriptional changes in human pluripotent stem cells after stresses of a variety of genotoxic agents, not only IR exposures.

## CONCLUSION

In summary, human pluripotent stem cells display unique molecular and gene expression features defining both their self-renewal and pluripotent capabilities, and high propensity to undergo cell death upon moderate to severe genotoxic stress exposures. The apoptotic mode of cell death appears to be the main driving force clearing damaged human pluripotent stem cells from stressed cell populations. Whereas, the high efficacy of DNA repair, and robust induction of antioxidant and/or pro-survival pathways at the level of altered global gene expression in cells that are destined to recover after genotoxic stress may play a primary role in protecting a subpopulation of human pluripotent stem cells from death and transfer of damaged genetic material to progeny. Future directions in studying human pluripotent stem cells should ask if these surviving cells carry any "molecular memory", or molecular changes associated with prior genotoxic stress exposure. In the planning, evaluation, and subsequent implementation of human pluripotent stem cell-based research activities, detailed gene expression analyses integrated with other global "omics" approaches will undoubtedly inform future basic science, cell regenerative-based and disease modeling studies.

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