Radiation Quality Effects On Transcriptome Profiles In 3-D Cultures After Charged Particle Irradiation

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INTRODUCTION

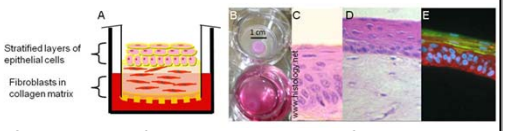
In this work, we evaluated the differential effects of low- and high-LET radiation on 3-D organotypic cultures in order to investigate radiation quality impacts on gene expression and cellular responses. Current risk models for assessment of space radiation-induced cancer have large uncertainties because the models for adverse health effects following radiation exposure are founded on epidemiological analyses of human populations exposed to low-LET radiation. Reducing these uncertainties requires new knowledge on the fundamental differences in biological responses (the so-called radiation quality effects) triggered by heavy ion particle radiation versus low-LET radiation associated with Earth-based exposures. In order to better quantify these radiation quality effects in biological systems, we are utilizing novel 3-D organotypic human tissue models for space radiation research. These models hold promise for risk assessment as they provide a format for study of human cells within a realistic tissue framework, thereby bridging the gap between 2-D monolayer culture and animal models for risk extrapolation to humans. To identify biological pathway signatures unique to heavy ion particle exposure, functional gene set enrichment analysis (GSEA) was used with whole transcriptome profiling. GSEA has been used extensively as a method to garner biological information in a variety of model systems but has not been commonly used to analyze radiation effects [1]. It is a powerful approach for assessing the functional significance of radiation quality-dependent changes from datasets where the changes are subtle but broad, and where single gene based analysis using rankings of fold-change may not reveal important biological information.

METHODS

2-D Cell Culture

Normal immortalized human esophageal epithelial cells (EPC2-hTERT) were cultured in Keratinocyte Serum Free Media and FEF3 cells (normal human esophageal fibroblasts) were cultured in DMEM (10% FBS+ pen/strep).

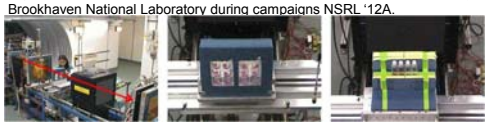
3-D Organotypic Culture



Organotypic model of human esophageal epithelium. Cultures were grown in DMEM followed by Epidermalization media (DMEM, Ham's F12 and supplements). (A) Schematic diagram of an organotypic culture showing individual layers; (B) Stromal-equivalent collagen/fibroblast base prior to epithelial seeding., shown in 24 mm transwell inserted into a 6-well plate; (C) H&E stained section of human esophagus (image from *www.histology.net*); (D) H&E stained section of a formalin-fixed paraffin-embedded slice from a 15-day organotypic culture of EPC2-hTERT cells grown in our laboratory at JSC; (E) A section from a similar culture immunostained for the basal marker KRT14 (red) and the suprabasal marker KRT13 (green); nuclei are counterstained with DAPI (blue).

Irradiation

Low-LET irradiation studies were conducted with a ^{137}cesium gamma source. High-LET particle was conducted at the NASA Space Radiation Laboratory at Brookhaven National Laboratory during campaigns NSRL '12A.



Experimental set-up and beam line at NSRL. Cells were irradiated in "track-segment mode" with the beam (arrow) perpendicular to the culture with constant LET across sample.

Gene Arrays and Analysis

Illumina platform (HT12 Expression Beadchip; Illumina, Inc.) arrays that provide coverage for 47,000 transcripts and splice variants were used to profile global gene expression changes in EPC2-hTERT epithelial cells grown in 3-D organotypic culture at 72 hrs post-exposure to 137Cs gamma-rays (100 rad) or 48Ti 350 MeV/u (30 rad) particle radiation. These exposures represent isotoxic doses based on clonogenic survival assays performed on cells cultured in 2-D monolayers. Raw Illumina data were preprocessed using R statistical package for background correction and normalization. GSEA (Gene Set Enrichment Analysis) was done using a t-test for ranking differentially expressed genes under gamma and titanium irradiation and run for three functional annotation datasets independently. These gene sets were obtained from the Molecular Signature Database (MsigDB), including curated gene sets, gene ontology gene sets, and oncogenic signature gene sets. The statistical significance of up- or down-regulated gene sets were assessed by permuting gene set 1,000 times as recommended in the GSEA reference manual. Gene sets were then ranked by two different approaches (FDR q-value based and leading-gene based ranking) and an average ranking was calculated for each gene set.

RT-qPCR

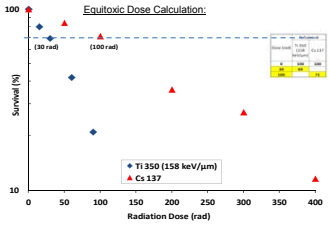
Total cellular RNA was isolated using Qiagen RNeasy spin columns from 2-D EPC2-hTERT cells cultured in KSFM and EPI or from 3-D epithelial layers. Fold changes in relative gene expression from triplicate samples were quantified using the comparative CT method with data normalized to GAPDH. Primers were ordered from SA Biosciences.

Clonogenic Survival Assay

EPC2-hTERT cells in sister flasks were irradiated and frozen. Upon thawing, cells were plated at increasing densities (n=3 plates per density). At 12 days, cells were fixed and stained with 0.1% crystal violet in 10% formalin and colonies containing more than 50 cells were counted.

RESULTS

Clonogenic Survival



➔ *Equitoxic cell death at 30r and 100r for Ti and Cs*

Up and Down Regulated Gene Sets

	Gene set name	FDR for γ-	FDR for 48Ti	New Rank
Common Gene Sets	BOSCO_INTERFERON_ANTIVIRAL_MODULE	0	0	1
	KERLEY_RESPONSE_TO_CISPLATIN_UP	0	0	2
	HUANG_DASATINIB_RESISTANCE_UP	4.14E-03	5.16E-03	3
	MISSIAGLIA_REGULATED_METHYLATION_UP	2.01E-04	0	3
	STAMBOLSKY_TARGETS_MUTATED_TP53_DN	0	0.0335	5
	INGA_TP53_TARGETS	0.0394	4.99E-03	6
	WIEDERSCHAIN_TARGETS_BMI1_AND_PCGF2	8.95E-03	0.0402	6
	HELLER_SILENCED_BY_METHYLATION_UP	4.40E-03	5.28E-03	6
	ALONSO_METASTASIS_EMT_UP	0.0209	0.0468	9
	AMUNDSON_DNA_DAMAGE_RESPONSE_TP53	8.37E-03	0.0316	9
	DORN_ADENOVIRUS_INFECTION_12HR_UP	0.0293	0.0315	9
Gamma-specific	SWEET_KRAS_TARGETS_UP	0.00276	0.3912	1
	GU_PDEF_TARGETS_UP	0	0.2103	1
	BEGUM_TARGETS_PAX1_FOXO1_FUSION_PAX3	0.00459	0.4944	3
	HANN_RESISTANCE_TO_BCL2_INHIBITOR_DN	0.00349	0.6028	4
	LE_SKI_TARGETS_UP	0.0122	0.3828	5
	GAURNIER_PSMD4_TARGETS	0.0154	0.4996	6
	CLAUS_PGR_POSITIVE_MENINGIOMA_DN	0.0371	0.7524	7
	DELYS_THYROID_CANCER_UP	0.00845	0.0708	8
	SCHOEN_NFKB_SIGNALING	0.00812	0.0896	8
	ST_INTERFERON_GAMMA_PATHWAY	0.0123	0.1337	10
	OZANNE_AP1_TARGETS_UP	0.00183	0.993	10
Titanium-specific	PHONG_TNF_TARGETS_UP	0.2092	0	1
	FINETTI_BREAST_CANCER_KINOME_RED	0.4429	0.0208	2
	DORN_ADENOVIRUS_INFECTION_48HR_DN	0.416	0.0307	3
	WANG_ESOPHAGUS_CANCER_PROGRESSION_UP	0.0611	0.0331	3
	LIN_SILENCED_TUMOR_MICROENVIRONMENT	0.5346	0	5
	DORN_ADENOVIRUS_INFECTION_24HR_DN	0.2677	0.0368	6
	WANG_METHYLATED_IN_BREAST_CANCER	0.4482	0.0199	6
	NUNODA_RESPONSE_TO_DASATINIB_IMATINIB_DN	0.1108	0.00333	6
	STREICHER_LSM1_TARGETS_DN	0.3293	0.0375	9
	HIRSCH_CELL_TRANSFORMATION_SIGN_UP	0.2691	0.0246	10

Table 1: MSigDB gene sets with top 10 rankings showing FDR (q-values) ≤0.05. Common (gamma plus titanium); gamma-specific and titanium-specific are listed. Full results of GSEA are shown in Table A1 in the Appendix. New rank score was derived by combining ranking based on q-value with lead gene based rank as described in the text.

Top-Ranking Data Sets from GSEA Analysis

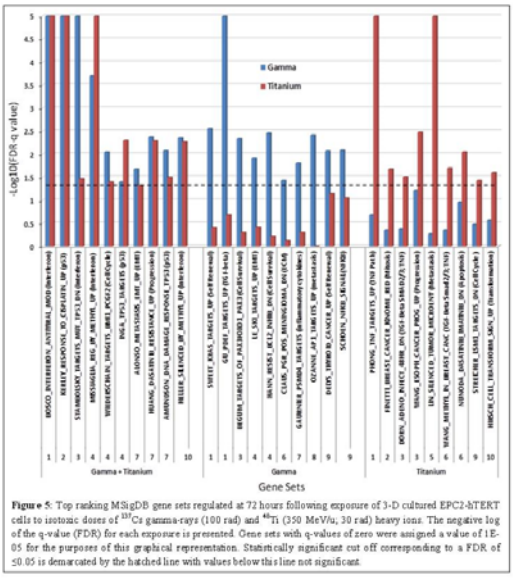
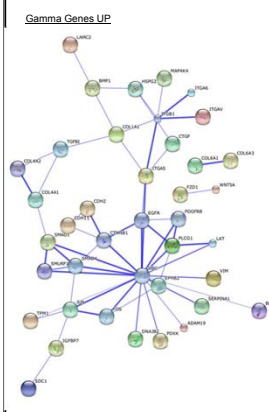


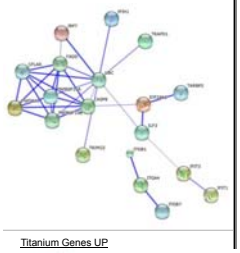
Figure 5: Top ranking MSigDB gene sets regulated at 72 hours following exposure of 3-D cultured EPC2-hTERT cells to isotoxic doses of ^{137}Cs gamma-rays (100 rad) and ^{48}Ti (350 MeV/u, 30 rad) heavy ions. The negative log of the q-value (FDR) for each exposure is presented. Gene sets with q-values of zero were assigned a value of 1E-05 for the purposes of this graphical representation. Statistically significant cut off corresponding to a FDR of ≤0.05 is demarcated by the hatched line with values below this line not significant.

Gene Interaction Networks

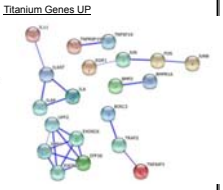
Gamma Genes UP



Gamma/Titanium Common Genes

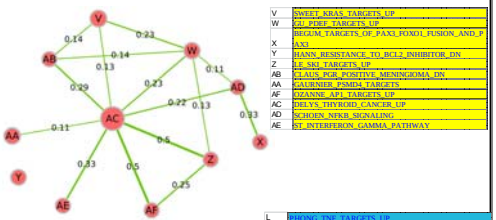


Titanium Genes UP



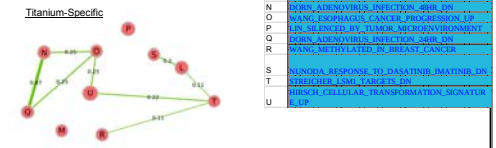
Enrichment Maps

Gamma-Specific



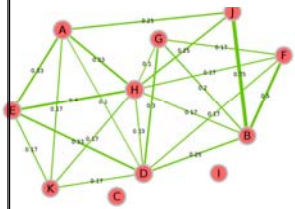
V	SWEET_KRAS_TARGETS_UP
W	GU_PDEF_TARGETS_UP
X	BEGUM_TARGETS_OF_PAX1_FOXO1_FUSION_AND_P AX3
Y	HANN_RESISTANCE_TO_BCL2_INHIBITOR_DN
Z	LE_SKI_TARGETS_UP
AB	CLAUS_PGR_POSITIVE_MENINGIOMA_DN
AA	GAURNIER_PSMD4_TARGETS
AF	OZANNE_AP1_TARGETS_UP
AC	DELYS_THYROID_CANCER_UP
AD	SCHOEN_NFKB_SIGNALING
AE	ST_INTERFERON_GAMMA_PATHWAY

Titanium-Specific



L	PHONG_TNF_TARGETS_UP
M	FINETTI_BREAST_CANCER_KINOME_RED
N	DORN_ADENOVIRUS_INFECTION_48HR_DN
O	WANG_ESOPHAGUS_CANCER_PROGRESSION_UP
P	LIN_SILENCED_BY_TUMOR_MICROENVIRONMENT
Q	DORN_ADENOVIRUS_INFECTION_24HR_DN
R	WANG_METHYLATED_IN_BREAST_CANCER
S	NUNODA_RESPONSE_TO_DASATINIB_IMATINIB_DN
T	STREICHER_LSM1_TARGETS_DN
U	HIRSCH_CELLULAR_TRANSFORMATION_SIGNATUR E_UP

Gamma/Titanium Common



Code	Gene set Name
A	BOSCO_INTERFERON_INDUCED_ANTIVIRAL_MODU LE
B	KERLEY_RESPONSE_TO_CISPLATIN_UP
E	STAMBOLSKY_TARGETS_OF_MUTATED_TP53_DN
D	MISSIAGLIA_REGULATED_BY_METHYLATION_UP
C	HUANG_DASATINIB_RESISTANCE_UP
F	INGA_TP53_TARGETS
G	WIEDERSCHAIN_TARGETS_OF_BMI1_AND_PCGF2
I	ALONSO_METASTASIS_EMT_UP
J	AMUNDSON_DNA_DAMAGE_RESPONSE_TP53
H	HELLER_SILENCED_BY_METHYLATION_UP
K	DORN_ADENOVIRUS_INFECTION_12HR_UP

CONCLUSIONS

- We identified 45 statistically significant gene sets at 0.05 q-value cutoff, including 14 gene sets common to gamma and titanium irradiation, 19 gene sets specific to gamma irradiation, and 12 titanium-specific gene sets.
- Common gene sets largely align with DNA damage, cell cycle, early immune response, and inflammatory cytokine pathway activation. The top gene set enriched for the gamma- irradiated samples involved KRAS pathway activation, while the top ranking gene set identified for the titanium exposure contains genes whose expression is increased in TNF-treated cells (Phong_TNF_Targets_Up) [2]. TNF is a multifunctional, proinflammatory cytokine that controls diverse biological processes, dictating cell killing by activation of apoptotic programs but also acting as a strong survival signal through NFκB, p38, and JNK-dependent pathways.
- Another difference noted for the high-LET samples was an apparent enrichment in gene sets involved in cycle cycle/mitotic control. It is plausible that the enrichment in these particular pathways results from the complex DNA damage resulting from high-LET exposure where repair processes are not completed during the same time scale as the less complex damage resulting from low-LET radiation.

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REFERENCES

[1] El-Saghire et al (2013). *Int J Radiat Biol* 89:628-38.
[2] Phong et al (2010). *Mol Cell Biol* 30:3816-26.