Radiation Effects on Tissue Differentiation in a 3-D Organotypic Aerodigestive Tract Model

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INTRODUCTION

The mucosa of the human aerodigestive tract is a typical stratified squamous epithelium where the outermost, luminal region consists of an epithelial cell compartment overlying a supportive stromal matrix. As the basal cells at the stromal interface divide and move upward, they become increasingly committed to terminal differentiation, lose the capacity to replicate, and are ultimately shed from the luminal surface. This process is fed by a population of basal cells with stem-like characteristics that serve as a reservoir for maintenance of the normal tissue structure. In the esophagus, cell fate determination controlling normal tissue homeostasis is dependent on intercellular Notch signaling (Ohashi 2010). This evolutionarily conserved pathway is important in embryonic development and differentiation as well as in angiogenesis, apoptosis, proliferation, stem cell renewal, and epithelial-mesenchymal transition (EMT). The outcome of Notch activity is dependent on cellular framework and is contingent on the timing and levels of pathway activity. In the esophagus, studies suggest a tumor suppressive function of Notch via promotion of differentiation and suppression of cancer-promoting EMT. In this study, we investigated the effects of radiation exposure (100 cGy 137Cs gamma-rays) on differentiation in a 3-D organotypic esophageal model using immunohistochemistry for differentiation markers (CDH1, IVL, KRT13 and 14) combined with focused gene expression analysis using qRT-PCR for Notch-specific pathway effectors and downstream targets. Qualitative changes in tissue morphology and distribution of differentiation markers were not observed in the irradiated compared to unirradiated samples at 3, 5 and 7 days following exposure. However, qRT-PCR analysis revealed significant upregulation of Notch pathway genes and downstream effectors that were consistent with an enhanced terminal differentiation in irradiated cultures. Although the exact role of these genes in the radiation response in this tissue remains to be elucidated, one possible explanation is that activation of a differentiation program following radiation exposure functions as a protective mechanism to eliminate genetically damaged cells from the replicating cell population.

Ohashi et al., 2010. Gastroenterology 139(6): 2113–23.

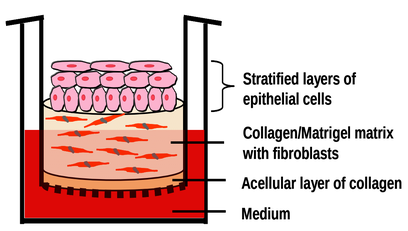
METHODS

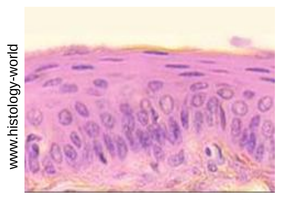
2-D Cell Culture

Normal immortalized human esophageal epithelial cells (EPC2-hTERT [1]) were cultured in Keratinocyte Serum Free Media (KSFM) for growth and in Epidermalization media (EPI, with DMEM, Ham's F12 and supplements) for micronucleus assays (unless otherwise indicated). FEF3 cells (normal human esophageal fibroblasts) were cultured in DMEM (10% FBS+ pen/strep).

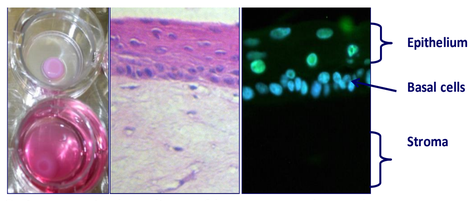
3-D Organotypic Culture

3-D cultures consisted of FEF3 fibroblasts in a layer of collagen I and Matrigel. EPC2-hTERT cells were seeded on top of this matrix and cultured in EPI media. Creation of an air-liquid interface triggered differentiation and stratification of the epithelial layer. Total culture time was 14-16 days.





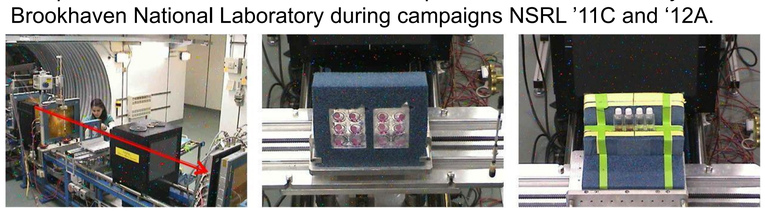
H&E section of normal stratified squamous esophageal epithelium.



3-D organotypic culture of human esophageal cells grown at Johnson Space Center.

Irradiation

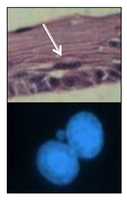
Low-LET irradiation was conducted with a ^{137}cesium gamma source. High-LET particle was conducted at the NASA Space Radiation Laboratory at Brookhaven National Laboratory during campaigns NSRL '11C and '12A.



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.

Cytokinesis-Block Micronucleus Assay

Following irradiation, cells were fed with media containing cytochalasin B to inhibit cytoplasmic division and allow for accumulation of binucleated cells and micronuclei (MN) [2]. For the 3-D assay, cultures were irradiated one day prior to airlift. Cells were harvested 65-70 hours post-irradiation.



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% NBF and colonies containing more than 50 cells were counted.

RT-qPCR

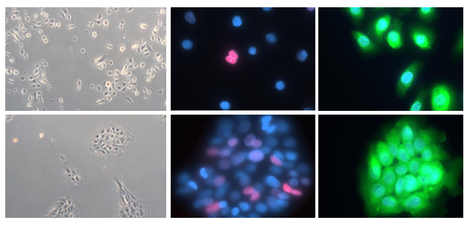
Total RNA samples was isolated using Qiagen RNeasy spin columns from EPC2-hTERT cells cultured in epidermalization (EPI) media or keratinocyte serum free media (KSFM) at 72 hours post-irradiation. Fold changes in relative gene expression from triplicate samples were quantified using the comparative CT method with data normalized to GAPDH .

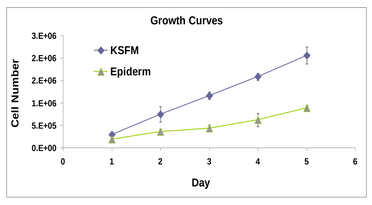
Immunocytochemistry

Monolayer cultures were fixed in 4% paraformaldehyde and immunostained with rabbit antibody to p21 (BD Biosciences) and Alexa594 labeled secondary antibody (Invitrogen). Nuclei were counterstained with DAPI.

RESULTS

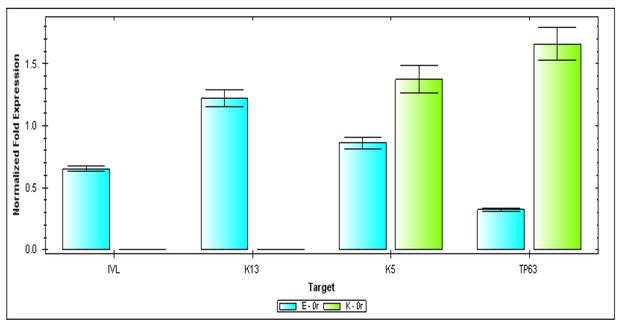
2-D Differentiation of EPC2-hTERT Cells





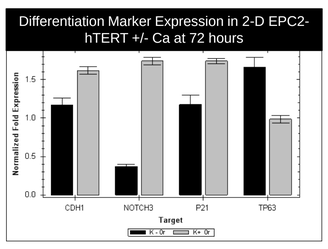
- *Clustered cell growth in EPI with evidence of stratification, vs. dispersed monolayer growth in KSFM*
- *Slower population growth in EPI media*
- *KSFM cultures represent proliferating basal population*

2-D Media Comparison: Differentiation Markers

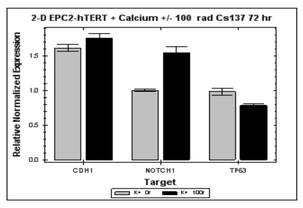


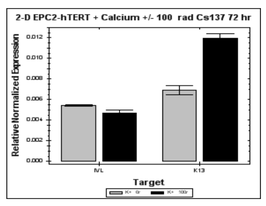
- *Markers of epithelial cell late differentiation (Involucrin, K13) are observed in cells grown in EPI media but not KSFM*
- *Markers of basal-like cells (K5, TP63) are expressed in cells grown in both EPI and KSFM with higher levels in KSFM cultures*

IR Effects on Differentiation Markers in 2-D EPC2-hTERT

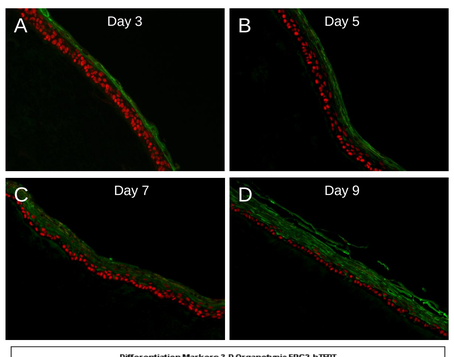


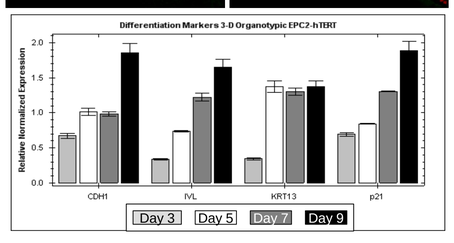
- *Calcium treatment enhanced expression of the differentiation markers CDH1, Notch 3, and p21 while p63 expression was suppressed.*
- *IR exposure enhanced expression of CDH1, Notch 1, and KRT13 while p63 expression was suppressed and no change observed for IVL.*





Differentiation in 3-D Organotypic Culture



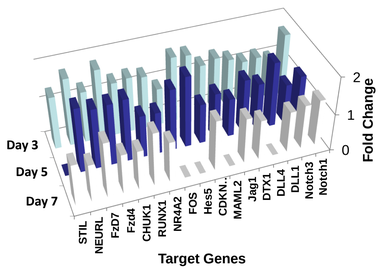


Proliferative basal population fuels tissue stratification. Cells become increasingly committed to terminal differentiation as they move upwards through epithelial layer and are ultimately shed from the luminal surface.

Tissue differentiation modeled in 3-D organotypic culture of EPC2-hTERT esophageal epithelial cells. Cultures were harvested at day 3 (panel A), day 5 (panel B), day 7 (panel C) and day 9 (panel D) post epithelial seeding and fixed in formalin and processed for histology. Top panel shows immunostaining of sections (6 µm) with antibodies against p63 (red) and involucrin (green). Bottom panel: Real time qPCR analysis of differentiation genes in RNA harvested from sister cultures. Data are ddCt values normalized to the housekeeping genes GAPDH and RPL13.

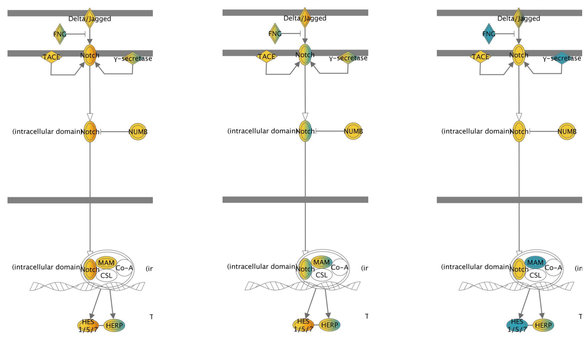
Notch Signaling Pathway RT PCR Array (SA Biosciences)

3D EPC2-hTERT 100r γ-ray		Day 3	Day 5	Day 7
Notch Signaling				
Notch Receptors	Notch1	1.72	1.21	1.23
	Notch3	1.12	1.06	1.18
Notch Ligands	DLL1	1.23	1.26	2-
	DLL4	1.23	1.26	2-
	Jag1	1.49	1.03	1.21
	DTX1	1.37	1.46	1.06
Transcription Cofactor	MAML2	1.38	1.27	1.14-
Notch Target Genes				
Cell Cycle	CDKN1A	1.74	1.07	1.35
Transcription Factors	Hes5	1.77	1.91	1.1-
	FOS	1	1.66	1.13-
	NR4A2	1.42	1.13	1.08
	RUNX1	1.48	1.14	1.43
Immune	CHUK1	1.43	1.68	1.05
Pathways that Crosstalk with Notch				
Wnt Signaling	Fzd4	1.87	1.64	1.06
	FzD7	1.37	1.61	1.48

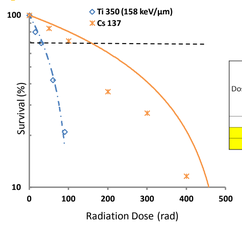


- *Small perturbations observed in mRNA expression of Notch receptor and ligands, as well as target genes that implement Notch signals. Coincide with alterations in Wnt receptor expression.*

Dynamic role of Notch signaling pathway



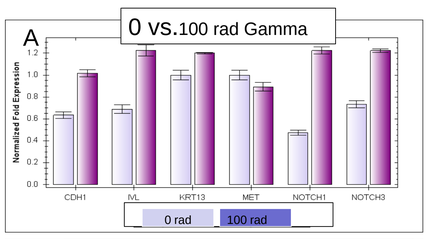
Equitoxic Dose Calculation – 2D Clonogenic Survival

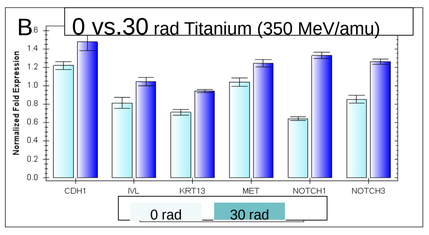


Dose (rad)	% Survival	
	Ti 350 (158 keV/µm)	Cs 137
0	100	100
30	69	
100		71

70

Radiation Effects on Markers of Differentiation in Organotypic Culture





- *Gamma-rays and HZE particles irradiation both result in increased expression of differentiation markers at 72 hours post-exposure*
- *Upregulation of Notch genes provides a clue to mechanism of radiation impact on cellular differentiation in this model*

CONCLUSIONS

Notch signaling pathway perturbed following radiation exposure with both γ-rays and heavy ions; in both 2-D and 3-D cultures of EPC2-hTERT cells.

Functional significance of Notch alterations unknown. Possible considerations are enhanced differentiation, or cell survival functions of this pleiotropic pathway.

Future studies to determine activation state of notch proteins following radiation, combined with pathway inhibitors will refine these preliminary results. Investigate role of p53.

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REFERENCES

[1] Okawa et al., Genes and Development 2007, 21: 2788-2803.
[2] Fenech. Nature Protocols 2007, 2: 1084 - 1104.