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Radiation-Induced Notch Signaling in Breast Cancer Stem Cells

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Abstract

Purpose—Breast cancers are thought to be organized hierarchically with a small number of breast cancer stem cells able to self-renew and to regrow the entire tumor. Importantly, breast cancer stem cells are resistant to established chemotherapeutic agents and relatively resistant to radiation. Self-renewal of breast cancer stem cells is under control of the Notch signaling pathway, which suggests that targeting this pathway could be a novel way of eliminating breast cancer stem cells. The γ -secretase complex controls the final intramembranous cleavage step that activates Notch receptors upon binding of its ligands and specific inhibitors of γ -secretase, which prevent Notch activation, are currently clinically tested against solid cancers in combination with radiotherapy.

Radiation activates Notch signaling and therefore, we sought to explore patterns of Notch receptor and ligand expression in response to radiation that could be crucial in defining optimal dosing schemes for γ -secretase inhibitors if combined with radiation.

Methods and Materials—Using MCF-7 and T47D breast cancer cell lines we used realtime RT-PCR to study the Notch pathway in response to radiation.

Results—We show that Notch receptor and ligand expression during the first 48 hours after irradiation followed a complex radiation dose-dependent pattern and was most pronounced in mammospheres, enriched for breast cancer stem cells. Additionally, radiation activated the Notch pathway. Treatment with a γ -secretase inhibitor prevented radiation-induced Notch family gene expression and led to a significant reduction in the size of the breast cancer stem cell pool.

Conclusions—Our results indicate that, if combined with radiation, γ -secretase inhibitors may prevent up-regulation of Notch receptor and ligand family members and thus reduce the number of surviving breast cancer stem cells.

Keywords

Breast cancer stem cells; Notch; Radiation

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Introduction

Pre-clinical [1] and clinical data [11] support that breast cancers are organized hierarchically with a small number of radio- and chemotherapy-resistant cancer stem cells (CSCs) able to self-renew, regrow the tumor after sublethal treatment, and give rise to all of the differentiated progeny found in the initial tumor. Since the progeny of CSCs lack these features, cancer can only be controlled if all CSCs are eliminated. The observation that CSCs are resistant to established anti-cancer therapies including radiation [2,17,21,30] has led to numerous efforts aiming to find novel agents that target CSCs specifically.

In breast cancer, CSCs can be prospectively identified using antibodies against surface proteins [10], based on ALDH activity [11], or lack of proteasome function [27]. For the latter, we recently described an imaging system that allows for easy detection and tracking of CSCs. It is based on the stable expression of a green fluorescent protein, ZsGreen, fused to the C-terminal degron of murine ornithine decarboxylase, cODC. In cells with active 26S proteasomes this protein is translated and immediately degraded. CSCs which lack proteasome activity, accumulate the fluorescent fusion protein, ZsGreen-cODC [27].

The Notch signaling pathway controls the self-renewal of epithelial stem cells in the mammary gland [6], as well as the self-renewal of breast cancer stem cells (BCSCs) [8,22]. As such it seems to be a valid target for therapies directed against BCSCs [5]. This rationale is further supported by our previous data showing activation of Notch signaling in response to ionizing radiation [21] and data on glioma stem cells reporting that notch signaling mediates radioresistance [28].

Notch signaling relies on cell-cell interaction with a '*sending cell*' expressing Notch receptor ligands and a '*receiving cell*' expressing Notch receptors. The mammalian Notch receptor family consists of 4 different receptors, Notch-1-4. Notch receptors are trans-membrane proteins that undergo S1 cleavage in the Golgi apparatus. After export to the membrane and binding to its ligands Jagged-1 or -2, or DLL1, -3, or -4, the extracellular domain of Notch receptors is shed off (S2 cleavage), and the intra-membranous part is cleaved by the γ -secretase complex (S3 cleavage). This last cleavage step frees the intracellular domain (Notch-ICD, NICD) for nuclear translocation where it binds to the transcriptional repressor CBF-1 and turns it into a transcriptional activator for Notch target genes. The γ -secretase complex is sometimes called the proteasome of the membrane, and so far 91 different substrates of γ -secretase have been identified [15]. Because γ -secretase is also involved in the pathogenesis of Alzheimer's disease, the development of inhibitors is far advanced and has already led to the identification of compounds used in clinical trials. However, given the plethora of substrates it is very unlikely that effects of these inhibitors are restricted to Notch signaling.

Because the Notch pathway is one of the major pathways involved in self-renewal of breast [4,6,22] and glioma [13] CSCs, we sought to investigate expression changes in Notch receptors and ligands over time and in response to different, clinically relevant doses of radiation. Since Notch signaling is activated by radiation [21], knowledge about the temporal expression patterns of Notch receptor and ligand family members would be of particular importance if γ -secretase inhibitors are combined with radiation therapy.

Methods and Materials

Cell culture

Human MCF-7 and T47D breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). Human SUM159PT breast cancer cell line was

purchased from Asterand (Asterand, MI). MCF-7-ZsGreen-cODC and T47D-ZsGreen-cODC cell lines were generated as described in Vlashi et al. [27]. MCF-7 and T47D were cultured in log-growth phase in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) (supplemented with 10% fetal bovine serum and penicillin and streptomycin cocktail). SUM159PT was cultured in log-growth phase in F12 Medium (Invitrogen, Carlsbad, CA) (supplemented with 5% fetal bovine serum [Sigma Aldrich, St Louis, MO] and penicillin (100 units/ml) and streptomycin (100 µg/ml) cocktail [Invitrogen], insulin (5 µg/mL) and hydrocortisone (1 µg/ml)). Mammospheres were cultured in DMEM/F12 (1:1) (supplemented with BSA, B27, EGF, bFGF, heparin and a penicillin and streptomycin cocktail). All cells were grown in a humidified incubator at 37°C with 5% CO₂.

Irradiation

For gene expression experiments cells were irradiated at room temperature *using a* ¹³⁷Cs laboratory irradiator (Mark I, JL Shephard, San Fernando, CA) at a dose rate of 4.95 Gy/minute for the time required to apply a prescribed dose. For assessment of the number of breast cancer stem cells and *in vivo* experiments, irradiation was performed at room temperature using an experimental X-ray irradiator (Gulmay Medical Inc. Atlanta, GA) at a dose rate of 2.789 Gy/min for the time required to apply a prescribed dose. Corresponding controls were sham irradiated. Assessment of the number of BCSCs was performed 5 days after irradiation.

Animals

6–8-week-old female NOD scid gamma (NSG) mice were originally purchased from the Jackson Laboratories (Bar Harbor, ME) and re-derived re-derived, bred and maintained in a pathogen-free environment in the American Association of Laboratory Animal Care-accredited Animal Facilities of Department of Radiation Oncology, University of California (Los Angeles, CA) in accordance to all local and national guidelines for the care of animals. SUM159PT cells were injected subcutaneously into the thighs of 6-week old female NSG mice within Matrigel (BD Biosciences).

Immunohistochemistry for Notch-ICD staining of tumor sections

Tumors were removed from sacrificed mice, cut in half and snap frozen in liquid nitrogen, using Optimum Cutting Temperature (OCT) compound for embedding the tumor tissue. 7 µm thick frozen sections from the middle of the tumor were cut and placed onto microscope slides. The slides were fixed in ice-cold acetone for 5 minutes and left to air dry. The slides were rinsed with PBS/0.1% Tween-20, and then blocked with PBS/0.5% BSA/0.3% hydrogen peroxide/0.1% sodium azide/5 % goat serum for 30 minutes at room temperature (RT). The slides were then incubated with rabbit Cleaved Notch1 (Val1744) Antibody (Cell Signaling, Cell Signaling Technology, Inc. Danvers, MA) diluted 1:80 in PBS/0.5% BSA, overnight at 4°C. The next day the slides were washed twice with PBS/0.1% Tween-20, 5 minutes each, and then incubated with the secondary antibody, goat anti-rabbit-TRITC (Sigma) for 30 min at RT. The slides were washed twice with PBS/0.1% Tween-20, for 5 min each, and incubated with Hoechst 33342 (2 µg/ml in PBA solution) for 5 min at RT, followed by three washes in PBS at RT. One drop of Fluoromount with Anti-fade media was added on top of the sections and a coverslip was placed on top. The tumor sections were visualized using Olympus IX71 inverted fluorescent microscope at a magnification of 40x. For quantification of Notch-ICD-positive nuclei slides were visualized on a Keyence BZ9000 inverted fluorescence microscope at a magnification of 20x. Pictures were imported into the ImageJ software package (vs 1.47d). Co-localization of Notch-ICD and nuclei was performed using the 'co-localization finder' plugin. Four random fields of view were analyzed for each tumor.

Western Blot Analysis

The proteins were separated by SDS page and then transferred to polyvinylidene fluoride membranes (BioRad, Hercules, CA). Blots were blocked and incubated with a primary antibody against Notch-ICD (1:1,000, Cell Signaling Technologies, Danvers, MA) overnight at 4°C. Membranes were washed and incubated with a secondary horseradish-peroxidase-conjugated antibody for 40 minutes at room temperature. Proteins were detected using the ECL plus Membrane Blot Analysis detection system (GE Healthcare).

Confocal Microscopy

MCF7 monolayer and mammosphere cells were harvested, dissociated, and counted as a single cell suspension. Cells underwent radiation treatment as described previously, fractionated for five days or single doses, and were cytopun onto glass slides. Cells were fixed using 4% paraformaldehyde for 10 min at room temperature and then washed once for five minutes using TBS. Cells were permeabilized by covering cells with TBS + 0.2% TritonX100 for 10 minutes at room temperature. Again cells were washed with TBS, three times for five minutes. Cells were stained with a primary rabbit anti-Notch-1-ICD antibody at a dilution of 1:100 in PBS + 1% BSA for 40 minutes at 4°C. Cells were washed 3 x five minutes with TBS and TRITC-conjugated secondary antibody was added at 1:100 dilution for 30 minutes in the dark at 4°C. CD44 expression was visualized using anti-CD44 FITC-conjugated antibody (BD Bioscience, Sparks, MD). Following an additional wash in TBS, cells were counter stained with the nuclear stain DAPI. Cells were again washed with TBS, allowed to dry in the dark for 20 minutes and coverslips were then mounted using fade-resistant gel. Slides were analyzed under a fluorescent microscope.

Drug treatment

In gene expression experiments the α -secretase inhibitor XVII (WEP-III-31-C, EMD Bioscience) was applied 1 hour before irradiation. In experiments assessing the number of breast cancer stem cells, the inhibitor was added 1 hour before irradiation and thereafter once daily over the 4 following days at a concentration of 5 μ M. Controls were treated with solvent only (DMSO 0.01%).

Quantitative Reverse Transcription-PCR

Quantitative PCR was performed in the My iQ thermal cycler (Bio-Rad, Hercules, CA) using the 2x iQ SYBR Green Supermix (Bio-Rad). C_t for each gene was determined after normalization to GAPDH or RPLP0 and C_t was calculated relative to the designated reference sample. Gene expression values were then set equal to 2^{-C_t} as described by the manufacturer of the kit (Applied Biosystems). All PCR primers were synthesized by Invitrogen and designed for the human sequences of human *Notch-1*, *2*, *3*, and *4*, *DLL-1*, *3*, and *4*, *Jagged-1* and *2*, *RPLP0*, and *GAPDH*.

Statistics

Unless indicated otherwise, at least three biological independent experiments were performed. A p -value equal to or less than 0.05 was considered to indicate a statistical significant difference. All data is represented as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error mean (SEM).

Results

Radiation activates Notch signaling

First, we tested if radiation activates Notch signaling in breast cancer. Using MCF-7 breast cancer cells we confirmed that radiation activated Notch1 after a single radiation dose of 3

Gy (data not shown), or a fractionated treatment of 5×3 Gy (Figure 1a). Indeed, radiation induced localization of NICD into the nucleus of irradiated cells, which could only be weakly detected in un-irradiated cells.

In order to test if the translocation of NICD into the nucleus affected activation of Notch target genes, we transfected MCF-7 cells with a luciferase reporter for CBF-1 [7,29]. Four hours after treatment with a single dose of 3 Gy, CBF-1 activation was comparable to the activity seen after stimulation with a synthetic DLL4 peptide (Figure 1b) mtCBF1-Luc: Control, 0.45, SEM 0.05; DLL4, 0.80, SEM 0.50; $p=0.293$; 3Gy, 0.90, SEM 0.30; $p=0.062$; wtCBF1-Luc: Control, 1.51, SEM 0.61; DLL4, 4.91, SEM 1.68, $p=0.030$; 3Gy, 3.98, SEM 0.32, $p=0.003$). Western Blot analysis confirmed activation of Notch1, 3 hours after exposure of MCF-7 cells to either 0 or 3 Gy of radiation (figure 1c).

In order to test if radiation activates Notch signaling in vivo, we implanted SUM159PT cells into the flanks of NOD scid gamma (NSG) mice. Tumors were irradiated with a single dose of 3 Gy. Four hours after irradiation the tumors were explanted and fresh-frozen sections were stained with an antibody against Notch-ICD. The staining confirmed that radiation activates Notch in vivo (figure 1 d).

Radiation induces the expression of Notch receptors and ligands

MCF-7 breast cancer cells were cultured as monolayer cultures or as mammospheres. Under the latter culture conditions differentiated cells die from anoikis, thus resulting in mammosphere cultures enriched for BCSCs [21,23]. Cells from monolayer or mammosphere cultures were irradiated with 0, 2, 4, 6, or 8 Gy. mRNA was extracted 0, 1, 3, 6, 12, 24, and 48 hours later and subjected to qRT-PCR using primers specific for the Notch receptors 1–4, and the Notch receptor ligands DLL1, -3, -4 and Jagged1 and Jagged2 (Figure 2a–c, Supplementary Table 1). Radiation-induced expression of Notch family member genes was most clearly observed in mammospheres. Among the Notch receptors, mRNA of the Notch2 receptor was consistently up-regulated over the entire range of radiation doses applied, while Notch3 receptor expression was only observed early after doses of 2 or 4 Gy (Figure 2a). DLL1 expression was increased after 2 or 4 Gy, while induction of DLL3 expression was only seen after higher doses of 6 or 8 Gy (Figure 2b). Expression of Jagged1 was only observed after 2 Gy or 8 Gy (Figure 2c). When the experiment was repeated with T47D breast cancer cells, a different pattern of radiation-induced gene expression was seen, though the time course of induction was similar. (Figure 2d).

γ -secretase inhibition prevents radiation-induced expression of Notch receptors and ligands

Next, we sought to test if treatment with a γ -secretase inhibitor would alter the radiation-induced expression pattern of Notch receptor and ligand family members. MCF-7 mammospheres were treated with a γ -secretase inhibitor or solvent, and irradiated with 0, 2, 4, 6, or 8 Gy. mRNA was extracted 0, 1, 3, 6, 12, 24, and 48 hours later, reverse transcribed and amplified using specific primer pairs for Notch receptor and ligand family members most profoundly induced by radiation (Figure 2a–c). Treatment with the γ -secretase inhibitor completely abrogated radiation-induced gene expression of Notch2, DLL1, -3, and Jagged1 (Figure 2a–c).

γ -secretase inhibition prevents radiation-induced increases in BCSCs

We had previously reported that ionizing radiation increased the number of breast cancer stem cells [17,21] and that this increase coincided with activation of Notch [21]. In order to demonstrate that breast cancer cells with low proteasome activity are enriched for BCSCs we performed a limiting dilution assay in vivo. Sum159PT cells were sorted based on

proteasome activity and injected into immune-compromised mice. SUM159PT-ZsGreen-cODC⁺ cells showed increased tumorigenicity consistent with enrichment for breast cancer stem cells (figure 3a). To test if inhibition of Notch prevented the radiation-induced increase in the number of breast cancer stem cells, we irradiated MCF-7-ZsGreen-cODC and T47D-ZsGreen-cODC breast cancer cells with 0, 2, 4, 6, or 8 Gy in the presence or absence of a γ -secretase inhibitor (figure 3b). Consistent with our previous data, radiation caused a dose-dependent increase in the percentage of ZsGreen-cODC-positive breast cancer stem cells. This increase was partially prevented by treatment with γ -secretase inhibitor. More importantly, drug treatment significantly reduced the absolute number of BCSCs (Figure 3c).

The reduction in the absolute number of BCSCs was reflected in a reduced sphere-forming capacity of γ -secretase inhibitor-treated MCF-7 cells but not in T47D cells (figure 3c). However, when we combined radiation with γ -secretase inhibitor treatment, T47D BCSCs were sensitized to radiation (figure 3d).

Discussion

Inhibitors of the γ -secretase complex are now increasingly being used as anticancer agents and are currently studied in a large number of clinical trials against breast cancer, colorectal cancer, glioma, melanoma, and lung cancer. The rationale for introducing these compounds into breast cancer treatment regimens is based on studies reporting overexpression of Notch receptors in breast cancers [3,19,25] and a central role for the Notch signaling pathway in the self-renewal process of breast cancer stem cells [6]. Furthermore, preclinical use of γ -secretase inhibitors reduced the number of breast cancer stem cells [20,24,25] and partially prevented radiation-induced reprogramming of non-tumorigenic breast cancer cells into breast cancer stem cells [16].

However, a recent paper from the Woodward lab reported that Notch inhibition by the γ -secretase inhibitor RO4929097 led to increased self-renewal and radioprotection of BCSCs [26] thus, suggesting that timing of a γ -secretase inhibitor treatment could be a critical parameter in the treatment design when combined with radiation therapy. A possible explanation for the radioprotective effect of RO4929097 might be proliferative effect of this γ -secretase inhibitor in breast cancer, potentially affecting radiation sensitivity in an unfavorable manner similar to EGFR inhibitors in colorectal cancer [3]. In our experiments we observed radiosensitization of T47D cells under sphere forming conditions. However, in MCF7 cells the γ -secretase inhibitor was very toxic and the sphere-forming capacity of MCF7 cells was drastically decreased in the presence of the compound. As a consequence of the normalization step, the γ -secretase inhibitor seemed to protect MCF7 cells from radiation but since the number of spheres formed dropped substantially, which in our eyes did no longer allow for proper interpretation of the data.

Mouse studies suggest that Notch receptor and ligand expression and Notch activation are highly dynamic [25] and part of the general cellular response to oxidative stress [21,24]. Therefore, knowledge of the exact temporal expression pattern of the Notch receptor and ligand family members appears to be crucial for the design of effective treatment schedules.

Consistent with our previous findings of radiation-induced activation of Notch signaling [21], we report here a complex pattern of radiation-induced expression of Notch receptor and ligand family members after exposure of breast cancer cells to single fractions of ionizing radiation. Even though we also demonstrate radiation-induced activation of CBF-1, the downstream target of Notch, it is important to remember that canonical Notch signaling requires cell-cell contact between a signal-sending and a signal-receiving cell. This suggests

that Notch signaling pattern may follow a different scheme than the expression of its signaling pathway components and will most likely be complicated further by radiation-induced cell death. With hypofractionated radiotherapy regimen in mind, it was remarkable that DLL3, a divergent Notch ligand family member that does not activate Notch signaling in adjacent cells but inhibits Notch receptors when expressed in the same cell [14], was consistently induced after higher doses of 6 and 8 Gy, suggesting that signaling relevant for breast cancer stem cells at these large fractions differs from signaling seen after conventional fractions of 2 Gy. This was consistent with our previous report on radiation-induced reprogramming of non-tumorigenic breast cancer cells into breast cancer stem cells, which showed an optimum reprogramming capability after single doses of 4 Gy, while the cell killing effects of single doses of 8 Gy or 12 Gy of radiation became prominent over the reprogramming effects of radiation [16].

Our observation that Notch inhibition prevented radiation-induced Notch receptor and ligand gene expression was consistent with data published by other groups reporting dependence of Notch gene expression on Notch signaling [12] and data reporting that Notch mediates radioresistance in GBM [28].

We were the first to describe that radiation enriches for relatively radiation-resistant and chemoresistant BCSCs [21] and we and others have reproduced these findings using several different marker systems for BCSCs [17,30]. The experiments in our current study show that the percentage of BCSCs with low proteasome activity increased 5 days after irradiation. Radiation itself inhibits the proteasome [18] and so do some chemotherapeutic agents [9]. However, this effect is only transient and does not explain increased numbers of BCSCs with low proteasome activity at later time points or gain of BCSCs traits [16,17]. The finding that γ -secretase inhibitors prevent induction of Notch family member gene expression, enrichment for BCSCs, and decrease sphere-forming capacity [20] suggest that combining radiation and γ -secretase inhibitors could be a useful approach against breast cancer.

Conclusions

In summary, we conclude that radiation in the clinically relevant dose range, induces rapid and lasting changes in Notch receptor and ligand family member gene expression. Combining γ -secretase inhibitors and radiotherapy will require carefully designed treatment schedules to ensure synergism of both treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a.

b.

Figure 1. Radiation activates Notch signaling

(a) MCF-7 cells were irradiated with 5 daily doses 3 Gy. After the last fraction, cells were fixed and stained with antibodies against the active form of Notch1 (NICD) and CD44, and counterstained with Hoechst 33342. Confocal imaging microscopy revealed that radiation caused an accumulation of NICD in the nuclei, indicating activation of the Notch pathway. (b) MCF-7 cells were stably transfected with a CBF-1-luc reporter construct or a luciferase gene with a mutated binding motif for CBF-1. Treatment of the cells with a synthetic DLL4 peptide or irradiation with a single dose of 3 Gy both led to significant activation of the Notch pathway. (c) Western Blot Analysis of lysates from MCF-7 cells, 3 hours after exposure to 0 or 3Gy. Irradiation led to detectable level of activated Notch (Notch-ICD, Val1744). Equal loading was confirmed using an antibody against alpha-tubulin. (d) SUM159PT tumors were grown in NSG mice and irradiated with 0 (left) or 3 Gy (right). Fresh-frozen sections were stained with an antibody against Notch-ICD. Radiation led to a significant increase in nuclear translocation of activated Notch *in vivo* ($p=0.001$, unpaired two-sided t-test).

Figure 2a

Figure 2b

Figure 2c

Figure 2d

Figure 2. Radiation induces expression of Notch receptors and ligands

Seven days after plating, MCF-7 (a-c) or T47D (d) monolayer cultures or mammospheres were irradiated with 0, 2, 4, 6, or 8Gy. mRNA expression of Notch1, Notch2, Notch3, and Notch4, DLL1, DLL3, DLL4, Jagged1, and Jagged2, was analyzed after 0, 1, 3, 6, 12, 24, and 48h by qRT-PCR. For MCF7 mammospheres irradiation was repeated in the presence of a α -secretase inhibitor (right panels) for the time and dose points with significant radiation-induced changes. mRNA was extracted at indicated time points and gene expression of DLL1, DLL3, Jagged1, Notch2, was analyzed by qRT-PCR. ($n = 3$ for all data points, each performed in quadruplicates).

a.

b.

c.

Figure 3. Inhibition of the Notch pathway reduces the number of breast cancer stem cells

a) In vivo limiting dilution assay for SUM159PT-ZsGreen-cODC⁺ breast cancer stem cells and -ZsGreen-cODC⁻ breast cancer cells. ZsGreen-cODC⁺ cells consistently show increased tumorigenicity

b. and c.) MCF-7 and T47D cells were treated with a γ -secretase inhibitor 1 hour before irradiation with 0, 2, 4, 6, or 8 Gy. Drug treatment was repeated every 24 hours for 4 days. Five days after irradiation, cells were analyzed for the presence of ZsGreen-cODC⁺ breast cancer stem cells by flow cytometry. The percentage of ZsGreen-cODC⁺ breast cancer stem cells is shown in panel b). Panel c) shows the absolute number of ZsGreen-cODC⁺ breast cancer stem cells.

Table 1

Relative expression of DLL1, DLL3, DLL4, Jagged1, Jagged2, Notch1, Notch2, Notch3 and Notch4 RNA through the time (0,1, 3, 6, 12, 24 and 48h) after irradiation 2, 4, 6 and 8Gy in MCF-7 grown in monolayer and sphere culture conditions. Non irradiated control has been used as a reference. Means, S.D. and p values are shown.

	Time (h)	Monolayer												Mammospheres											
		2Gy						4Gy						6Gy						8Gy					
		Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value
DLL1	0	1.000	0.000		1.000	0.000		1.000	0.000		1.000	0.000		1.000	0.000		2.308	0.604		4.095	0.540		2.071	0.532	
	1	0.470	0.106	0.000	1.843	0.118	0.000	1.090	0.238	0.274	1.249	0.138	0.018	2.886	0.341	0.111	6.454	2.506	0.093	6.454	2.506	0.093	3.672	3.215	0.221
	3	0.343	0.227	0.004	0.941	0.047	0.046	0.567	0.052	0.000	0.695	0.343	0.099	9.006	0.387	0.000	6.553	0.175	0.001	10.371	4.599	0.039	8.735	1.578	0.001
	6	0.954	0.661	0.455	3.200	1.813	0.052	0.726	0.346	0.122	0.820	0.116	0.028	7.606	4.844	0.067	10.371	4.599	0.039	10.371	4.599	0.039	3.801	3.405	0.217
	12	0.155	0.110	0.000	2.477	3.387	0.246	0.360	0.191	0.002	0.105	0.026	0.000	6.011	2.579	0.036	14.698	8.066	0.043	14.698	8.066	0.043	3.033	0.085	0.018
	24	1.353	1.292	0.330	3.248	3.637	0.172	0.765	0.387	0.176	1.478	0.070	0.000	2.353	0.609	0.466	13.110	1.456	0.000	13.110	1.456	0.000	2.231	0.713	0.386
DLL3	48	0.575	0.417	0.076	2.728	2.217	0.124	0.353	0.186	0.002	0.722	0.036	0.000	1.811	0.169	0.121	10.400	6.992	0.097	10.400	6.992	0.097	1.713	0.732	0.266
	0	1.000	0.000		1.000	0.000		1.000	0.000		1.000	0.000		1.898	1.503		2.064	0.091		2.064	0.091		2.864	0.532	
	1	0.842	0.000	0.000	0.988	0.086	0.410	2.315	0.238	0.000	1.627	0.138	0.001	2.231	1.741	0.407	2.580	2.234	0.355	2.580	2.234	0.355	5.950	3.215	0.088
	3	0.734	0.377	0.144	0.351	0.021	0.000	0.249	0.052	0.000	0.862	0.343	0.262	2.258	1.779	0.401	2.331	1.899	0.410	2.331	1.899	0.410	6.808	1.578	0.007
	6	0.950	0.071	0.144	0.986	0.719	0.488	0.100	0.346	0.005	0.683	0.116	0.005	1.679	0.960	0.421	1.195	0.471	0.017	1.195	0.471	0.017	4.249	3.405	0.262
	12	0.877	0.033	0.001	0.780	0.310	0.144	0.396	0.191	0.003	0.255	0.026	0.000	1.869	0.993	0.490	2.363	1.928	0.401	2.363	1.928	0.401	4.940	0.085	0.001
DLL4	24	0.725	0.389	0.144	1.255	1.064	0.349	0.452	0.387	0.035	0.602	0.070	0.000	2.156	2.412	0.441	4.516	2.308	0.070	4.516	2.308	0.070	2.706	0.713	0.387
	48	0.620	0.654	0.186	0.642	0.655	0.199	1.148	0.186	0.120	2.188	0.036	0.000	2.524	2.156	0.350	0.960	0.056	0.000	0.960	0.056	0.000	2.570	0.732	0.302
	0	1.000	0.000		1.000	0.000		1.000	0.450		1.000	0.046		1.928	2.528		2.222	0.080		2.222	0.080		1.810	0.500	
	1	0.871	0.334	0.269	1.221	0.262	0.109	1.155	0.563	0.364	1.575	0.036	0.000	0.984	0.975	0.289	0.409	0.062	0.000	0.409	0.062	0.000	0.856	0.193	0.018
	3	1.188	1.347	0.411	1.046	0.529	0.443	1.007	0.004	0.490	1.122	0.239	0.217	0.877	0.007	0.256	0.403	0.414	0.001	0.403	0.414	0.001	0.548	0.277	0.009
	6	1.362	1.089	0.298	1.020	0.513	0.475	0.428	0.094	0.049	1.318	0.238	0.043	0.712	0.163	0.226	0.709	0.412	0.002	0.709	0.412	0.002	0.652	0.629	0.033
Jagged1	12	2.362	1.801	0.130	0.907	0.459	0.372	1.463	0.439	0.136	0.508	0.300	0.024	1.103	0.760	0.308	0.875	0.520	0.006	0.875	0.520	0.006	0.660	0.398	0.018
	24	2.017	1.438	0.144	2.199	2.446	0.222	0.916	0.372	0.408	1.158	0.103	0.037	0.602	0.645	0.214	0.466	0.179	0.000	0.466	0.179	0.000	0.933	0.830	0.096
	48	1.168	0.469	0.285	0.910	0.027	0.002	1.006	0.056	0.492	1.855	0.092	0.000	0.325	0.096	0.167	0.887	0.160	0.000	0.887	0.160	0.000	1.392	0.271	0.136
	0	1.000	0.000		1.000	0.000		1.000	0.000		1.000	0.000		8.054	8.980		2.334	1.398		2.334	1.398		2.801	0.987	
	1	1.367	1.378	0.334	1.646	1.436	0.240	1.815	2.142	0.273	1.108	0.674	0.398	22.899	9.525	0.060	4.448	2.966	0.163	4.448	2.966	0.163	2.972	0.000	0.390
	3	0.918	0.884	0.440	2.270	2.221	0.189	1.145	0.789	0.383	1.154	0.816	0.380	22.113	10.689	0.078	1.395	0.566	0.171	1.395	0.566	0.171	2.654	0.000	0.405

	Time (h)	Monolayer												Mammospheres																			
		2Gy				4Gy				6Gy				8Gy				2Gy				4Gy				6Gy				8Gy			
		Mean	SD	p value	p value	Mean	SD	p value	p value	Mean	SD	p value	p value	Mean	SD	p value	p value	Mean	SD	p value	p value	Mean	SD	p value	p value	Mean	SD	p value	p value	Mean	SD	p value	p value
Jagged2	6	0.822	0.320	0.195	0.292	1.359	1.047	0.292	0.406	1.276	1.122	0.346	0.346	28.589	8.387	0.022	0.022	2.233	1.216	0.464	0.250	3.343	0.794	0.250	0.250	8.732	7.347	0.283	0.283				
	12	0.280	0.398	0.018	0.195	1.305	0.548	0.195	0.492	0.358	0.422	0.029	0.029	23.173	5.848	0.035	0.035	2.140	0.806	0.423	0.202	3.488	0.807	0.202	0.202	6.091	4.789	0.467	0.467				
	24	1.279	0.550	0.215	0.347	1.565	2.318	0.347	0.459	1.030	0.348	0.445	0.445	23.646	9.661	0.055	0.055	1.838	1.150	0.330	0.497	2.793	1.713	0.497	0.497	14.897	9.400	0.096	0.096				
	48	1.196	0.838	0.353	0.479	1.031	0.940	0.479	0.359	1.212	0.970	0.362	0.362	15.588	10.035	0.194	0.194	1.685	1.441	0.303	0.321	2.013	2.525	0.321	0.321	3.851	0.000	0.199	0.199				
	0	1.000	0.000			1.000	0.000			1.000	0.000			0.445	0.280			1.323	0.724			1.326	0.834			2.719	1.468						
	1	0.542	0.014	0.000	0.004	1.683	0.249	0.004	0.422	3.221	0.323	0.000	0.000	0.741	0.467	0.200	0.200	0.928	0.507	0.241	0.476	1.371	0.863	0.476	0.476	4.552	2.458	0.165	0.165				
	3	0.440	0.029	0.000	0.000	1.297	0.017	0.000	0.000	4.524	0.364	0.000	0.000	0.443	0.279	0.496	0.496	2.107	1.153	0.187	0.282	0.954	0.600	0.282	0.282	4.232	2.286	0.195	0.195				
	6	0.169	0.006	0.000	0.000	0.381	0.038	0.000	0.000	1.543	0.042	0.000	0.000	0.235	0.148	0.158	0.158	0.893	0.488	0.221	0.112	0.571	0.359	0.112	0.112	2.513	1.357	0.433	0.433				
Notch1	12	0.416	0.029	0.000	0.000	0.936	0.093	0.151	0.419	0.366	0.019	0.000	0.000	0.481	0.303	0.444	0.444	1.491	0.815	0.401	0.086	0.476	0.300	0.086	0.086	3.519	1.900	0.297	0.297				
	24	0.470	0.017	0.000	0.002	0.658	0.096	0.002	0.000	2.049	0.218	0.001	0.001	0.508	0.320	0.406	0.406	0.948	0.518	0.253	0.417	1.487	0.936	0.417	0.417	1.025	0.553	0.067	0.067				
	48	0.399	0.018	0.000	0.005	0.819	0.068	0.005	0.000	1.829	0.190	0.001	0.001	0.358	0.225	0.348	0.348	1.062	0.581	0.326	0.423	1.192	0.751	0.423	0.423	4.350	2.349	0.183	0.183				
	0	1.000	0.000			1.000	0.000			1.000	0.000			1.330	1.258			0.992	0.887			1.467	0.512			1.387	0.726						
	1	0.761	0.233	0.075	0.306	1.125	0.394	0.306	0.000	1.812	0.829	0.083	0.083	1.015	0.589	0.357	0.357	2.379	0.360	0.033	0.362	1.347	0.208	0.362	0.362	1.268	0.340	0.405	0.405				
	3	0.207	0.138	0.000	0.316	0.773	0.760	0.316	0.000	1.203	1.283	0.399	0.399	1.759	0.068	0.293	0.293	1.344	0.935	0.330	0.245	1.793	0.540	0.245	0.245	1.639	0.039	0.291	0.291				
	6	0.222	0.100	0.000	0.125	1.832	1.071	0.125	0.070	1.800	0.604	0.042	0.042	1.512	0.340	0.410	0.410	0.912	0.735	0.455	0.412	1.376	0.425	0.412	0.412	1.775	0.197	0.211	0.211				
	12	2.446	1.669	0.104	0.097	1.413	0.459	0.097	0.248	2.050	0.316	0.002	0.002	1.903	0.697	0.264	0.264	0.307	0.237	0.133	0.014	0.443	0.137	0.014	0.014	1.285	0.403	0.420	0.420				
Notch2	24	2.073	1.154	0.091	0.152	0.662	0.498	0.152	0.064	0.639	1.338	0.332	0.332	0.548	0.623	0.195	0.195	0.333	0.264	0.143	0.010	0.315	0.152	0.010	0.010	1.007	0.359	0.231	0.231				
	48	2.140	0.904	0.047	0.012	0.474	0.260	0.012	0.017	0.775	0.543	0.257	0.257	2.126	1.815	0.283	0.283	0.757	0.295	0.343	0.320	1.309	0.170	0.320	0.320	1.280	1.048	0.445	0.445				
	0	1.000	0.000			1.000	0.000			1.000	0.000			1.630	0.236			1.430	0.263			1.700	0.137			1.210	0.726						
	1	0.870	0.409	0.306	0.466	0.940	1.133	0.466	0.054	0.770	0.323	0.143	0.143	1.300	0.335	0.118	0.118	2.180	0.938	0.127	0.435	1.800	0.980	0.435	0.435	2.640	0.340	0.018	0.018				
	3	0.340	0.523	0.047	0.170	0.520	0.769	0.170	0.022	0.110	0.364	0.007	0.007	2.980	1.342	0.081	0.081	5.320	5.574	0.147	0.272	2.380	1.770	0.272	0.272	3.500	0.039	0.003	0.003				
	6	0.510	0.741	0.158	0.272	0.790	0.550	0.272	0.006	0.070	0.042	0.000	0.000	4.790	3.963	0.120	0.120	4.910	3.556	0.083	0.000	16.440	2.288	0.000	0.000	4.900	0.197	0.001	0.001				
	12	0.200	0.162	0.001	0.120	0.680	0.402	0.120	0.002	0.360	0.019	0.000	0.000	10.450	7.255	0.052	0.052	4.900	3.180	0.066	0.005	12.740	4.188	0.005	0.005	6.060	0.403	0.000	0.000				
	24	0.840	0.906	0.387	0.459	1.110	1.748	0.459	0.033	0.200	0.218	0.002	0.002	5.050	4.947	0.149	0.149	2.170	2.460	0.316	0.325	1.390	1.088	0.325	0.325	2.630	0.359	0.019	0.019				
Notch3	48	1.530	0.720	0.136	0.203	1.850	1.589	0.203	0.014	2.170	0.190	0.000	0.000	1.880	2.696	0.440	0.440	0.960	0.293	0.054	0.488	1.730	1.556	0.488	0.488	1.120	1.048	0.454	0.454				
	0	1.000	0.000			1.000	0.000			1.000	0.000			2.020	1.454			2.810	1.657			0.530	0.439			1.820	0.957						
	1	0.820	0.310	0.186	0.138	2.090	1.499	0.138	0.024	2.040	0.866	0.053	0.053	2.370	2.014	0.410	0.410	4.830	4.408	0.249	0.151	0.850	0.163	0.151	0.151	1.750	0.545	0.459	0.459				

		Monolayer												Mammospheres											
		2Gy			4Gy			6Gy			8Gy			2Gy			4Gy			6Gy			8Gy		
		Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value
Notch4	Time (h)																								
	3	1.410	1.820	0.358	2.790	3.060	0.184	1.190	1.051	0.385	2.640	1.767	0.092	4.660	4.041	0.174	5.250	3.868	0.186	0.880	0.333	0.167	0.900	0.233	0.090
	6	1.470	1.762	0.334	0.800	0.395	0.215	1.290	1.017	0.324	2.160	0.228	0.000	4.180	3.733	0.202	3.230	4.329	0.441	0.790	0.155	0.194	0.920	0.499	0.111
	12	4.580	4.048	0.100	0.530	0.556	0.109	1.060	0.337	0.387	1.050	0.321	0.400	5.330	4.823	0.159	2.520	3.030	0.446	0.850	0.785	0.286	1.590	0.749	0.380
	24	0.880	1.024	0.425	0.460	0.615	0.102	1.230	0.591	0.269	2.390	0.355	0.001	2.580	1.958	0.356	1.990	1.164	0.261	0.950	0.130	0.094	0.480	0.672	0.059
	48	0.930	0.727	0.438	2.070	1.023	0.072	1.060	0.420	0.408	3.110	0.591	0.002	1.600	1.741	0.382	0.740	0.394	0.052	1.430	1.005	0.114	2.450	0.228	0.165
	0	1.000	0.000		1.000	0.000		1.000	0.000		1.000	0.000		1.330	1.258		0.992	0.887		2.350	1.398		0.470	1.398	
	1	0.761	0.233	0.075	1.125	0.394	0.306	3.380	2.142	0.063	3.910	1.436	0.012	1.015	0.589	0.357	2.379	0.360	0.033	10.420	2.966	0.007	1.160	2.966	0.367
	3	0.207	0.138	0.000	0.773	0.760	0.316	6.420	0.789	0.000	0.920	2.221	0.477	1.759	0.068	0.293	1.344	0.935	0.330	1.720	0.566	0.255	0.360	0.566	0.453
	6	0.222	0.100	0.000	1.832	1.071	0.125	0.200	0.614	0.043	0.090	1.047	0.103	1.512	0.340	0.410	0.912	0.735	0.455	4.810	1.216	0.041	1.120	1.216	0.288
12	2.446	1.669	0.104	1.413	0.459	0.097	0.050	0.052	0.000	0.080	0.548	0.022	1.903	0.697	0.264	0.307	0.237	0.133	3.240	0.806	0.197	2.020	0.806	0.086	
24	2.073	1.154	0.091	0.662	0.498	0.152	2.130	0.253	0.001	4.750	2.318	0.024	0.548	0.623	0.195	0.333	0.264	0.143	8.460	1.150	0.002	3.030	1.150	0.035	
48	2.140	0.904	0.047	0.474	0.260	0.012	1.140	0.996	0.410	8.970	0.940	0.000	2.126	1.815	0.283	0.757	0.295	0.343	2.310	1.441	0.487	6.160	1.441	0.004	