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DIFFERENTIAL EFFECTS OF PROTON THERAPY AND PHOTON THERAPY ON HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) POST-TREATMENT AGGRESSIVENESS

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NECK SQUAMOUS CELL CARCINOMA (HNSCC) POST-TREATMENT AGGRESSIVENESS.
Séminaire du Cancéropôle Provence-Alpes-Côte d'Azur, Jun 2017, SAINT-RAPHAEL, France. hal-
02382956

HAL Id: hal-02382956

<https://hal.science/hal-02382956v1>

Submitted on 4 Dec 2019

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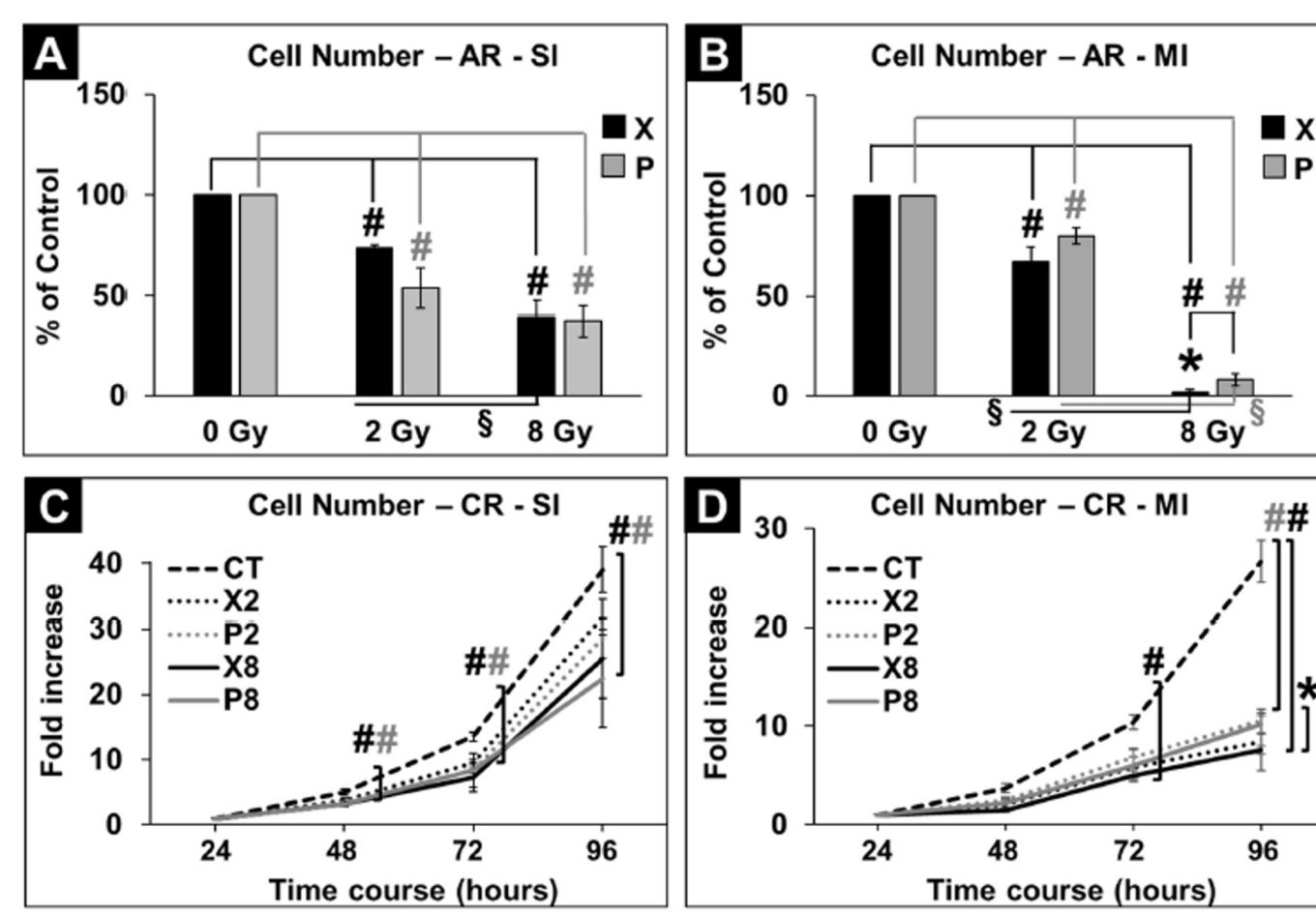
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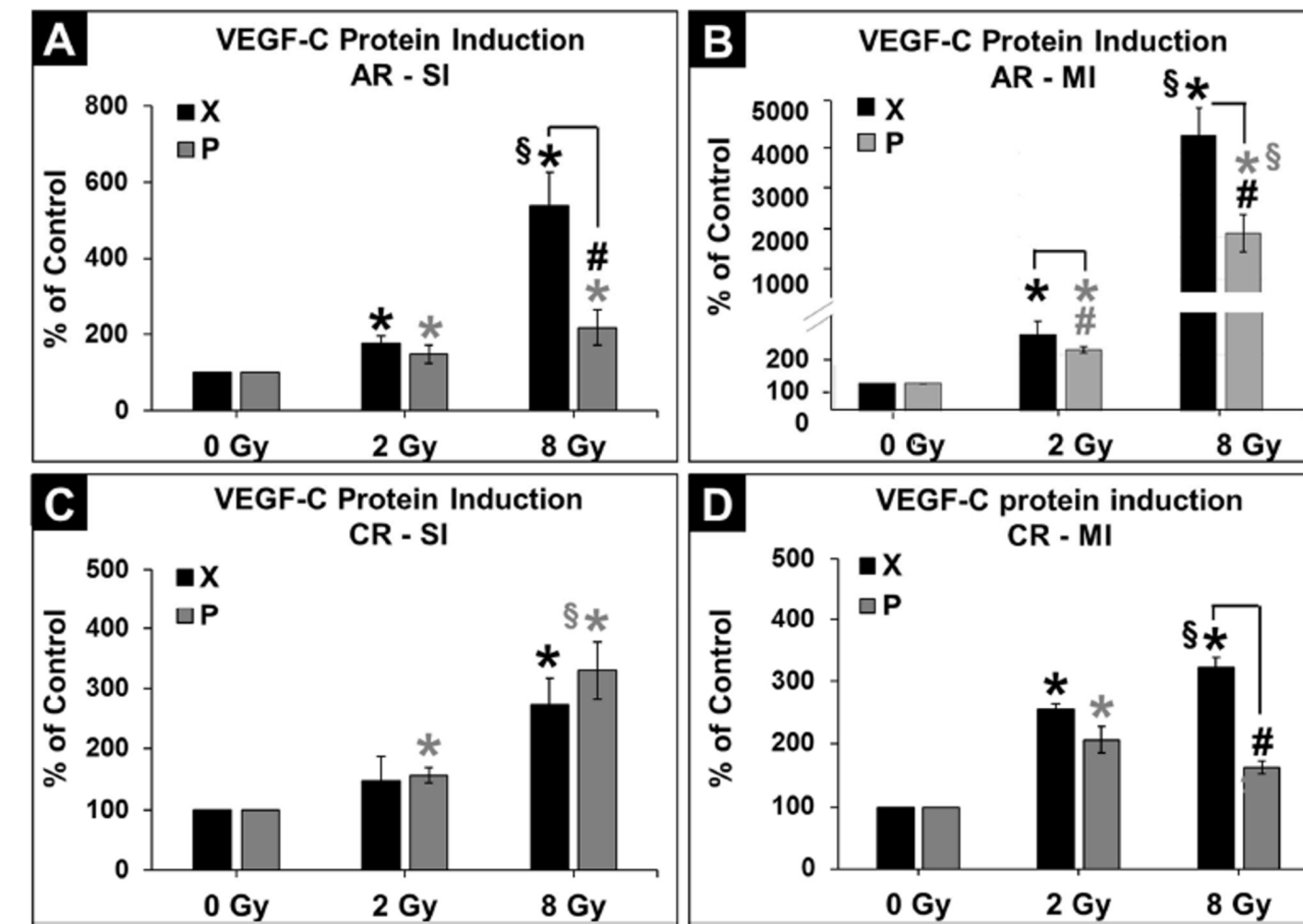
The treatment of squamous cell carcinoma of the aerodigestive tract (SCC) by standard radiotherapy is challenging because of the proximity of organs at risk. Due to its higher precision in tumor targeting, protontherapy could become the treatment of choice for SCC. Additionally, recent studies have shown that proton irradiation suppresses angiogenic genes and impairs tumor cell invasion/growth. We investigated the effectiveness of single and multiple proton (P) versus photon (X) irradiations in SCC cells on their proliferation potential and on the expression of genes / proteins involved in proliferation, (lymph)angiogenesis, metastasis and anti-tumor immunity.



CELL PROLIFERATION

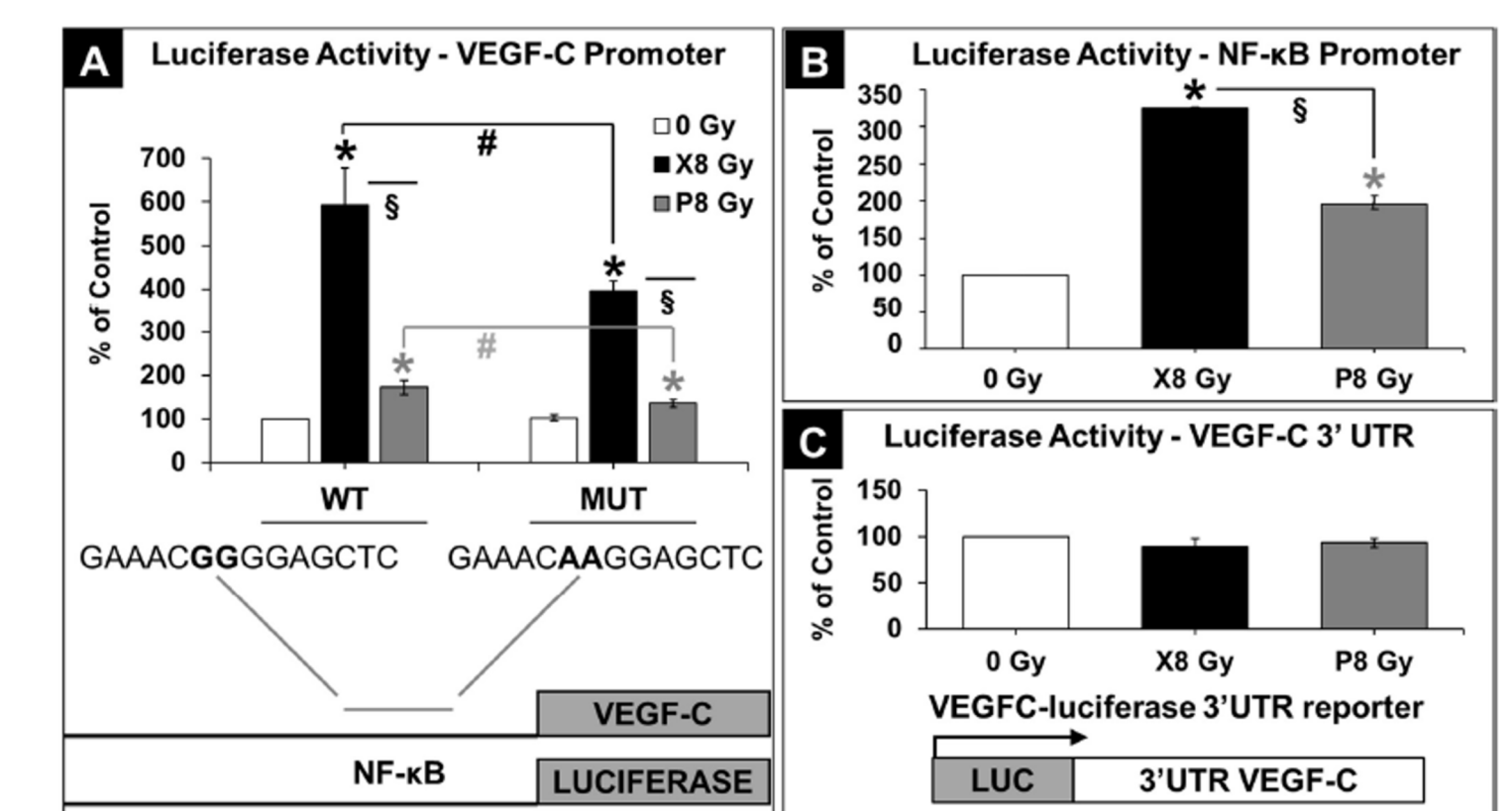
Cell proliferation after a single or multiple proton (P) or photon (X) irradiations at 2 Gy or 8 Gy.

Both P and X irradiations acutely and chronically affected cell growth and cell viability as measured by cell counting of viable cells 48, 72, 96 hours after cell seeding. $p < 0.05$



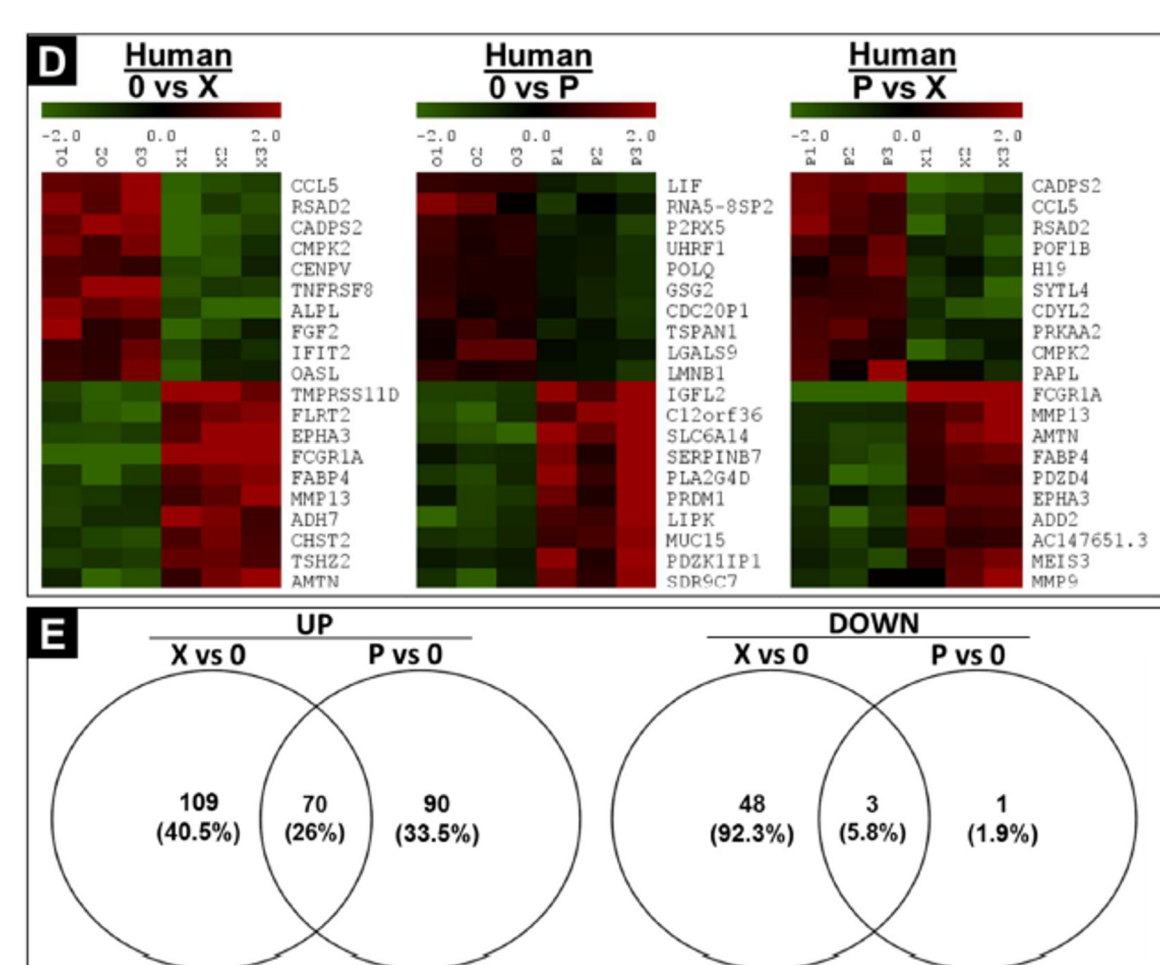
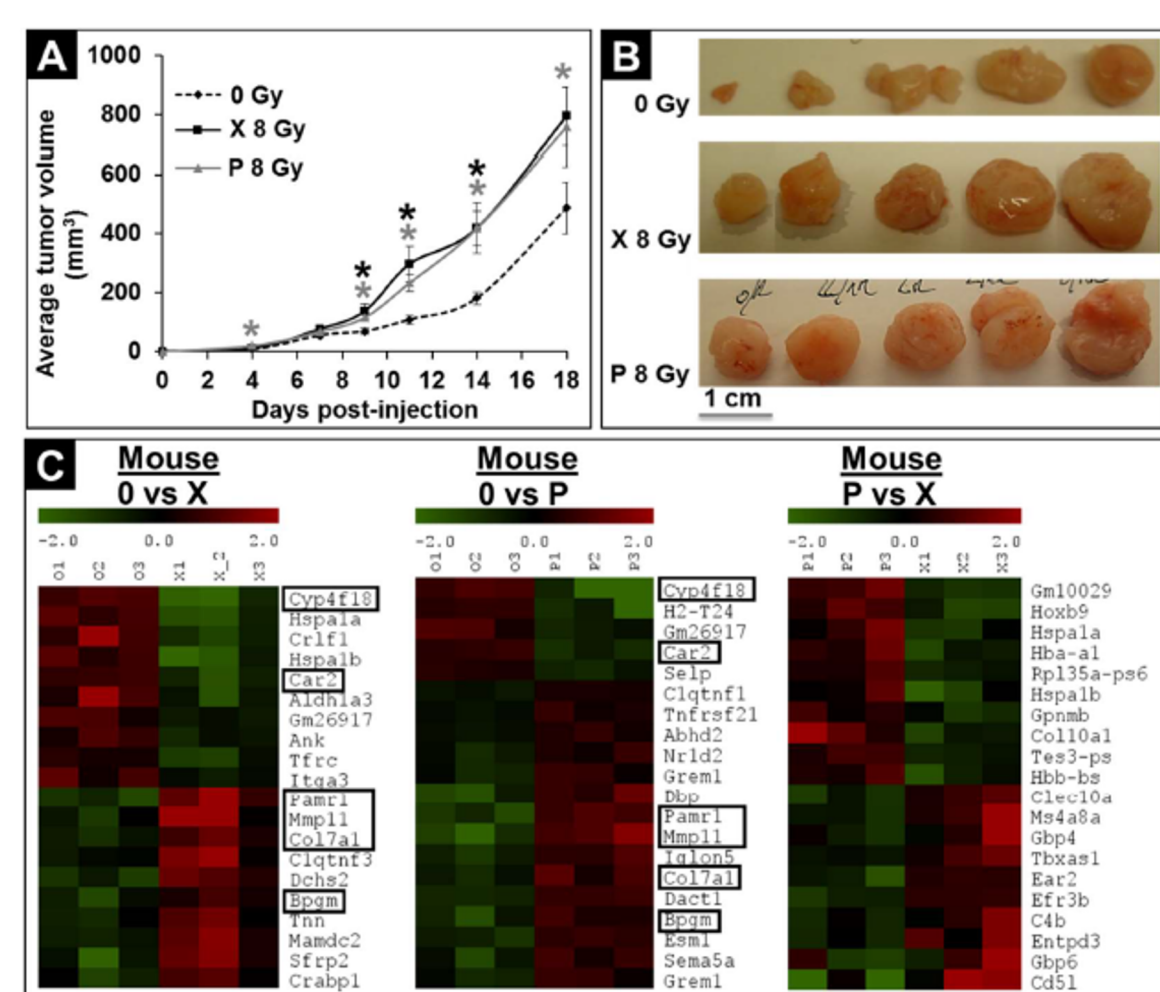
VEGF-C PROTEIN INDUCTION

In CAL33 cells, VEGF-C protein levels increased in a dose-dependent manner following both P and X irradiations. Furthermore, they were significantly lower after P irradiation, except for the CR-SI setting where the two types of radiations.



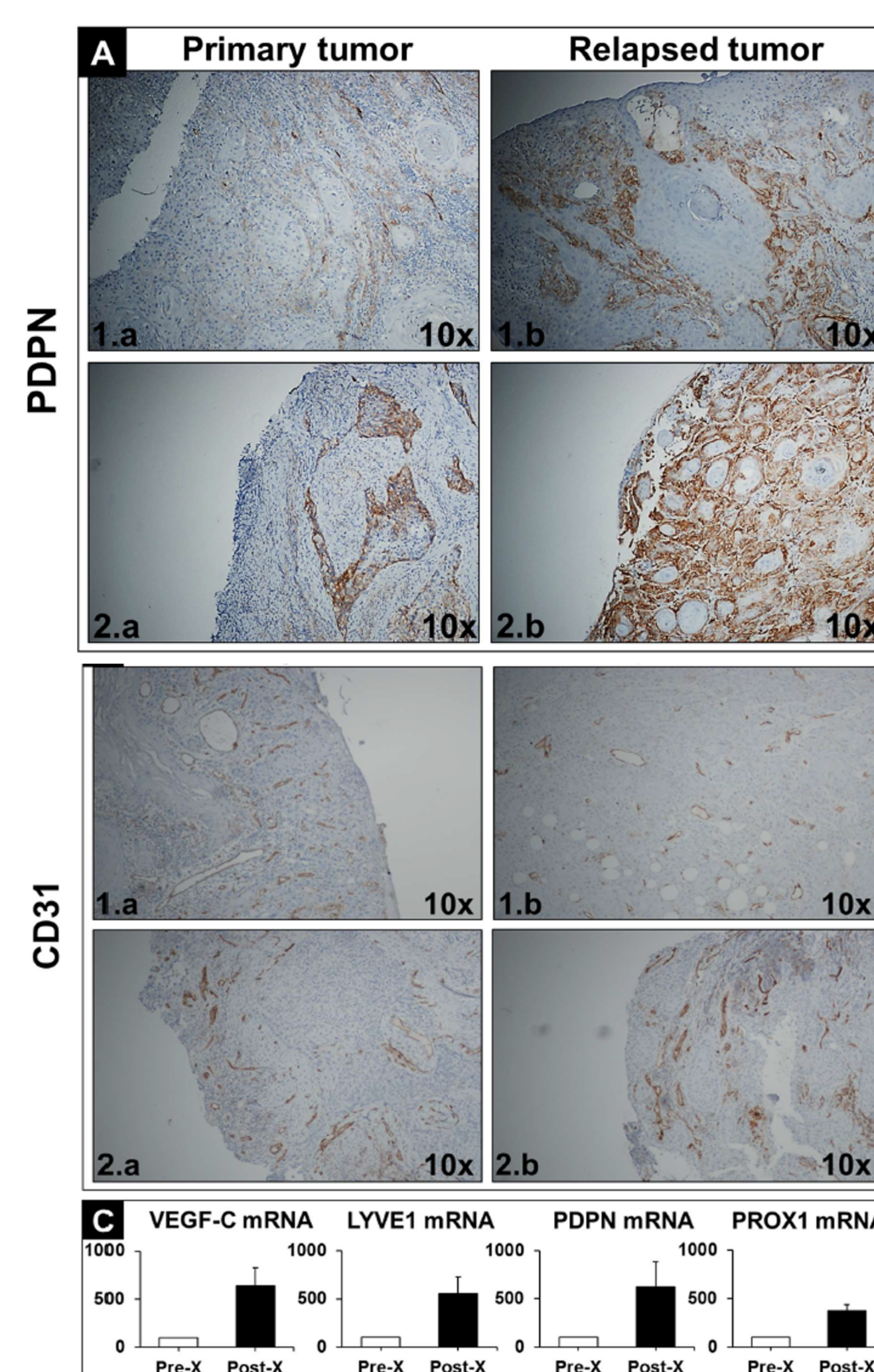
X AND P IRRADIATIONS STIMULATE VEGF-C PROMOTER ACTIVITY

Irradiation by either X or P stimulated the activity of the VEGF-C promoter especially in CAL33 cells surviving to multiple X irradiations (6- and 18-fold increase, respectively, $p < 0.001$). Mutation of the NF-kB binding site (MUT) had no effect on the basal VEGF-C promoter activity in non-irradiated cells. However, in cells surviving to MI by P and X, the activity of the MUT, as compared to WT, promoter was significantly decreased (by 33%, $p = 0.004$ and by 30%, $p = 0.027$, respectively).



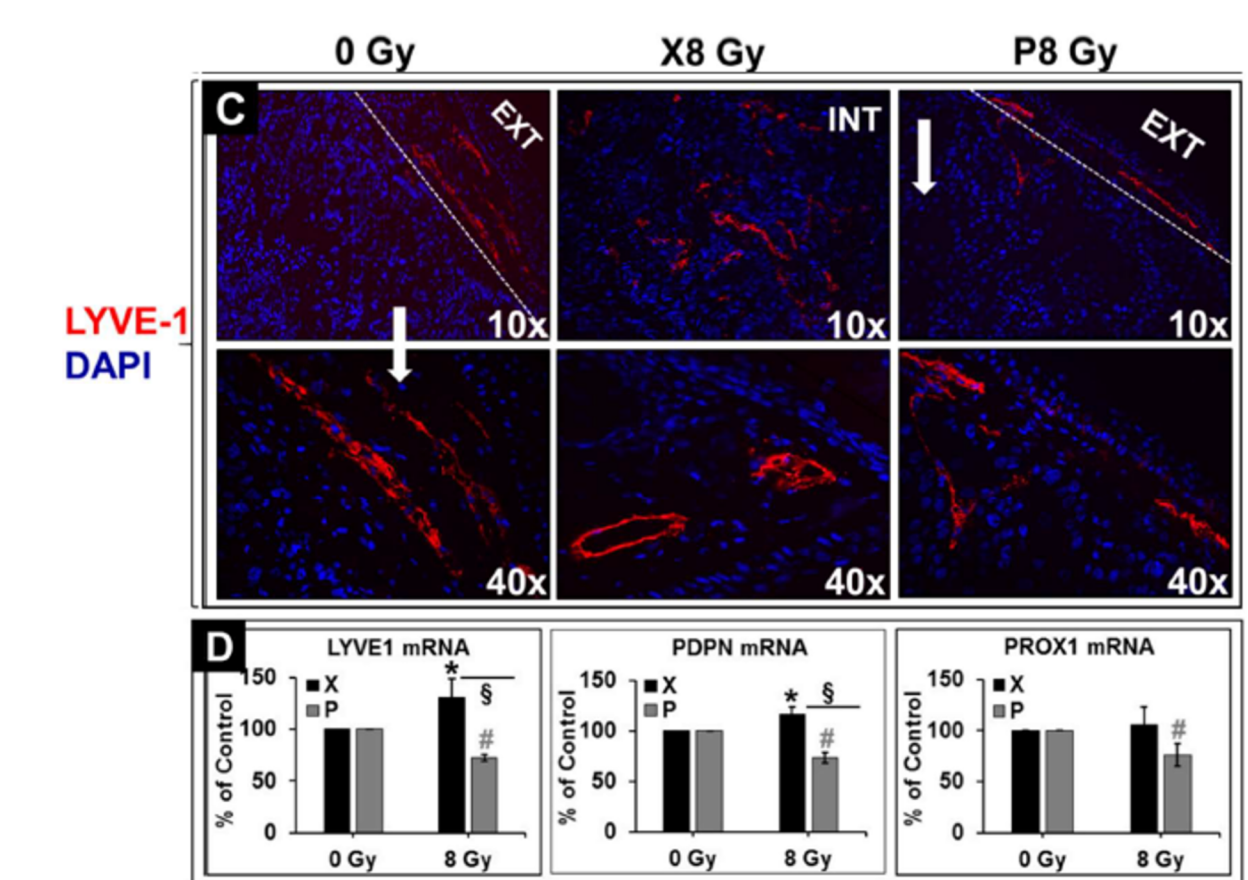
ANALYSIS OF TUMORS GENERATED BY SUBCUTANEOUS XENOGRAFTS

(A) Average tumor volume (mm³); (B) Representative images of tumor xenografts; (C) Heatmap of ten most up- and down-regulated mouse genes in tumors generated by non-irradiated cells vs P or X tumors, and in P vs X tumors; (D) Heatmap of ten most up- and down-regulated human genes in tumors generated by non-irradiated cells vs P or X tumors, and in P vs X tumors; (E) Venn diagrams showing common up-regulated and down-regulated human genes between P and X tumors. Framed genes are commonly expressed in P and X tumors. Selection is adjusted p value < 0.05 and $\text{loFC} > 1$.



EVALUATION OF LYMPHANGIOGENESIS IN BIOPSIES FROM HNSCC PATIENTS

Representative images of immunohistochemistry for (A) PDPN and (B) CD31 expression: (1) oral and (2) hypopharyngeal localization; Left panels (1.a, 2.a) – primary tumor; Right panels (1.b, 2.b) – relapsed tumor in the same patient after surgery and chemo-X radiotherapy (brown, PDPN/CD31; blue, hematoxylin - nuclei); (C) quantification of PDPN, VEGF-C, LYVE1 and PROX1 mRNA expression.



REPRESENTATIVE FLUORESCENCE IMAGES IN MURINE XENOGRAFTS

LYVE1 (lymphatic endothelial cells, red) / Hoechst (nuclei, blue), showing different patterns of lymphatic vessels development in X (both periphery and interior of the tumor), P and CT (periphery of the tumor) groups; dashed white lines delimit the tumor edge; CT, control (tumors generated by non-irradiated cells); (D) Murine LYVE1, PDPN and PROX1 mRNA levels in xenografts.

A mRNA levels (% of Control) - AR - SI				B mRNA levels (% of Control) - AR - MI							
Gene	2 Gy	8 Gy	2 Gy 8 Gy	Gene	2 Gy	8 Gy	2 Gy 8 Gy				
Lymphangiogenesis and metastasis	VEGF-A	122	159	143	160	VEGF-A	127	155	146	159	
	VEGF-C	130	227	159	209	VEGF-C	227	1206	146	159	
	VEGF-D	103	92	155	81	VEGF-D	124	209	124	159	
Inflammation	IL6	129	135	119	72	IL6	136	200	142	172	
	IL8	147	416	162	460	IL8	133	242	138	509	
Proliferation	CCL2	104	123	171	134	CCL2	117	112	109	149	
	TRP-2	89	112	102	102	TRP-2	121	78	105	144	
Anti-tumor immunity	Pd-L1	85	108	88	93	Pd-L1	173	151	151	141	
	PD-L1	108	103	91	112	Anti-tumor immunity	PD-L1	138	142	122	204
Gene score	4	6	8	3	Gene score	4	6	8	3		

QUANTITATIVE GENE EXPRESSION, AS PERCENTAGE OF CONTROL (0 GY), IN EITHER P OR X IRRADIATED CAL33 CELLS BELONGING TO (A) AR-SI, (B) AR-MI, (C) CR-SI AND (D) CR-MI GROUPS

Highlighted values – significantly different ($p < 0.05$) expression levels, as compared to control, for genes associated to favorable (dark grey) and non-favorable (black) outcomes; *, significantly different expression levels after low, as compared to high dose(s) of either P or X irradiation; #, significantly different expression levels after either low or high dose(s) of P, as compared to X irradiation.

CONCLUSIONS:

In vitro results:

- Protontherapy leads to lower expression of factors involved in (lymph)angiogenesis, inflammation and immune tolerance.
- Acquisition of less aggressive phenotype after protontherapy

In vivo results:

- VEGF-C mRNA levels increase in a dose-dependent manner and with the irradiation number, except in the cells surviving after three proton irradiations.
- Subcutaneous xenograft of proton-irradiated cells generate tumors with less lymphatic vessels than the tumors obtained by photon-irradiated cell xenograft

Patient studies:

- Relapsed tumors (after photontherapy) present a marked expression of genes involved in lymphangiogenesis/metastasis.

These observations suggest that photontherapy would lead to less pronounced lymphangiogenesis/metastasis, as compared to protontherapy.