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SUMMARY

Results produced under the SEPARATE project have been organized into two main topics relating to (i) the molecular mechanisms of out-of-target radiation effects in the hippocampus assayed through a multi-omics approach and (ii) the phenotypic and functional characterization of exosomes derived from irradiated mouse organs and their role in the mechanisms driving non-targeted effects. These results have been drafted into two manuscripts whose abstracts are provided hereafter.

In addition, a third manuscript reviewing the state of the art of the knowledge on the neurocognitive dysfunctions following radiotherapy, with focus on the mechanisms and therapeutic implications, has been recently submitted for publication in an upcoming special issue on "Animal models in Radiotherapy Research" in the journal *Cancers*. Notably, this work provided ground to frame the mechanistic investigations relative to the out-of-field radiation effects in the hippocampus currently developed under the SEPARATE project. The manuscript acknowledges SEPARATE funding, and the abstract is also reported.

Manuscript 1

Out-of-field hippocampus from partial-body irradiated mice displays changes in multi-omics profile and defects in neurogenesis

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Abstract

Our previous work, showing that brain and skin cancer development in genetically sensitive mice is increased by radiation exposure of distant tissues, highlighted the importance of communication between irradiated and non-irradiated tissues and organs in cancer induction. To test the potential contribution of “out-of-target” irradiation to non-cancer pathologies we have interrogated the hippocampus, a highly radiosensitive organ. Impairment of hippocampal neurogenesis is frequently observed after whole-brain exposure, and it is regarded as the most important mechanism of radiation-induced cognitive dysfunction. To test the impact of out-of-field irradiation on hippocampal neurogenesis we have evaluated the radiation-dependent modifications in the cellular composition of the subgranular zone (SGZ) of the dentate gyrus in a conventional mouse model. C57BL/6 female mice of 8 weeks of age have been whole body (WBI) or partial body (PBI) irradiated with 0.1 or 2 Gy of x-rays or sham irradiated (SI). PBI consisted in the exposure of the lower third of the mouse body, whilst the upper two thirds were shielded. Here we performed a multi-omics experimental setup and analyzed dentate gyrus tissue samples 15 days post-irradiation using metabolomics, proteomics, and microRNA profiling techniques. Metabolomic analysis at 15 days post-irradiation was able to detect spectral differences between 2 Gy WBI and 0.1 Gy WBI groups and controls but not between 2 Gy PBI and 0.1 Gy PBI groups and controls, indicating absence of early out-of-field radiation effects in the hippocampus. However, at 6 months postirradiation both WBI and PBI groups (2 Gy and 0.1 Gy) showed clear spectral differences compared to controls, with no discrimination between 2 Gy WBI and PBI groups. This spectral similarity was indicative of similar persistent metabolic effects occurring after WBI and PBI exposure. Proteomic analysis at 15 days postirradiation revealed a high number of deregulated proteins, i.e., 94 (0.1 Gy PBI), 66 (0.1 Gy WBI), 111 (2.0 Gy PBI), and 281 (2.0 Gy WBI) in the different treatment groups. Of these, 31 were commonly deregulated and the majority of these deregulated proteins was involved in synaptic transmission and memory-related processes. In addition, miRNome analysis revealed a marked overlap of miRNA expression profiles in WBI and PBI hippocampi, especially at high radiation dose. Notably these molecular alterations were accompanied by phenotypical changes in neurogenesis consisting of defects in the dynamic transition among neural stages in the dentate gyrus, pointing

to a complex disturbance in the control of progression of neural stem cells into neurons following high dose radiation exposure.

Altogether, our results demonstrate, for the first time, that the shielded hippocampus exhibits defects in multi-omics profile and alteration of neurogenesis similar to those induced by WBI. Our data may help to further identify and characterize molecular mechanisms governing the perturbation of adult hippocampal neurogenesis in response to radiation exposure with important implications in clinics and radiotherapy.

Manuscript 2

Phenotypic and functional characteristics of exosomes derived from irradiated mouse organs and their role in the mechanisms driving non-targeted effects.

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Abstract:

Molecular communication between irradiated and non-irradiated neighbouring cells initiates radiation-induced bystander effects (RIBE) and out-of-field (abscopal) effects. RIBE and abscopal effects are characterised by DNA damage, and each is an example of the non-targeted effects of ionising radiation (NTE). Another well-known NTE, radiation-induced genomic instability (RIGI), can be described as delayed damage responses within the progeny of irradiated and non-irradiated cells. The relatively new mechanism identified for mediating NTE is exosomes, which are small (30–120 nm of diameter) membrane vesicles of endosomal origin. They are secreted by normal and pathological cells into the microenvironment. Upon internalisation, they release their bioactive cargo molecules, which can change molecular profile, signalling pathways and gene regulation in the recipient cells. Some well-known examples of those signalling pathways include WNT, HH and Notch signalling in developmental biology and ERK1/2, p38 MAPK, TGF- β pathways in the context of cancer biology. Changes in terms of “gene regulation” include for example downregulation of immune function related genes by tumor derived exosomes.

Here we investigated exosome-mediated radiation signalling *in vitro* by focusing on changes in exosome characteristics, as well as changes in calcium signaling, cell viability, DNA damage and biochemical profile upon transfer of exosomes from 2Gy x-ray whole body irradiated (WBI) or partial body irradiated (PBI) mice to recipient mouse embryonic fibroblast (MEF) cells. In parallel, 0Gy irradiated mice were used as controls. In this study, we focused on brain, liver, heart and plasma, which were analysed at two time points, 24 hours and 15 days.

There was no significant change in exosome size as indicated by the distribution of exosome diameters. However, the yield of exosomes from both organs and plasma increased dramatically after both WBI and PBI. Treatment of MEF cells with the exosomes derived from organs and plasma resulted in significantly decreased cell viability and increased DNA damage as shown by γ H2AX immunostaining, comet assay and chromosomal analysis data at both the 24 hour and 15 day post irradiation time points. In addition, increased calcium signalling and altered biochemical profile were observed in the recipient MEF cells.

Keywords: Exosomes ; Ionizing radiation; Non Targeted Effects

Manuscript 3

Neurocognitive Decline Following Radiotherapy: Mechanisms and Therapeutic Implications

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Abstract

The brain is exposed to ionizing radiation in a number of clinical situations, particularly during radiation therapy for treatment of malignant central nervous system tumors. Cranial radiation therapy has been associated with the risk of long-term cognitive morbidity. The damaging effects of ionizing radiation on the brain strongly depend on age at exposure, with younger age correlating with larger deficits. Radiation has been shown to induce alterations in several cellular compartments in the mouse brain. Indeed, brain exposure causes a dysfunction of the neurogenic niche due to alterations in the neuronal and supporting cell progenitor signaling environment, particularly in the hippocampus – a region of the brain critical to memory and cognition - with subsequent alterations in the rate of neurogenesis, neural differentiation and apoptosis, culminating in neuron decline and long-term consequences for cognitive functions. In addition to neural stem cells, mature neural cells and glial cells are known targets of irradiation. We will review the current knowledge about radiation-induced damage in stem cells of the brain and discuss potential treatment interventions and therapy methods to prevent and ameliorate radiation related cognitive decline.

Keywords: Neural stem cells; Ionizing radiation; Neurocognitive effect