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Functional analysis of head and neck cancer exosomes released in response to ionizing radiation

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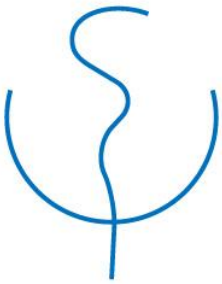

Doctoral thesis

TECHNISCHE UNIVERSITÄT MÜNCHEN

Chair of radiation biology

Faculty of Medicine

Functional analysis of head and neck cancer exosomes released in response to ionizing radiation

Lisa Mutschelknaus

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Summary

Ionizing radiation is able to damage biologically relevant cellular structures and induces a damage response network in targeted cells. Previous findings demonstrate that also non-targeted cells without any direct energy deposition respond to radiation exposure. It was suggested that cell-to-cell communication via gap-junctions, soluble factors or extracellular vesicles is responsible for the observed effects. Especially, the impact of exosomes, a class of smaller-sized extracellular vesicles is studied extensively. Exosomes are endosomal secreted vesicles that consists of a bilayer lipid membrane and transport incorporated proteins, RNA as well as DNA fragments to recipient cells. Recent evidence demonstrates that exosomal cell-to-cell signaling plays a major role in the cellular stress response. Moreover, exosomes drive oncogenesis and promote tumor progression as well as metastasis in many cancer types.

This doctoral thesis addresses the question if ionizing radiation affects the exosomal signaling of squamous head and neck cancer. For this purpose, the effect of radiation on exosome release and uptake rates, exosomal composition and the impact on recipient cells including phenotypic alterations and intracellular signaling cascade changes were investigated.

The results described in this thesis highlight that the cell-to-cell signaling via exosomes is a component of the radiation stress response of head and neck cancer cells. Hereby, radiation increases the exosome release and uptake, accompanied by changes in the exosomal protein composition. Moreover, exosomes from irradiated donor cells increase the survival, DNA DSB repair, wound healing capacity and chemotaxis-induced motility of recipient cells. This may promote a more radioresistant and invasive phenotype. Beyond that, radiation-induced exosomal transfer between different cell types induces a repair response or wound healing, suggesting a highly complex interaction within an irradiated tissue containing heterogeneous tumor and non-tumor cell populations.

The obtained results further demonstrate that the endocytic uptake of exosomes derived from irradiated cells activate the intracellular AKT-signaling in the recipient cells. In line bioinformatics analyses assign several deregulated exosomal proteins to migration processes and the AKT-signaling pathway. Together these results

suggest that the exosomal components are functional upon transfer to the recipient cells. The simultaneous AKT-inhibition (5 µM Afuresertib) and exosome transfer further show the relevance of intracellular AKT-pathway activation in radiation- and exosome-mediated migration induction. Apart from the protein cargo, nucleic acids might also contribute to the functionality of exosomes since treatment with RNase blocks the exosome-accelerated DNA repair efficiency.

In conclusion, exosomes are shown to be biologically active components involved in the head and neck cancer radiation response. The new insights from this thesis demonstrate that head and neck cancer exosomes may deliver a protective and potentially tumorigenic message to recipient cancer cells or neighboring non-cancer tissues. As a consequence, exosomes may act as drivers of radioresistance and metastatic head and neck cancer progression during tumor radiotherapy and are therefore an attractive target to improve radiation therapy strategies.

Zusammenfassung

Ionisierende Strahlung ist in der Lage, biologisch relevante Zellstrukturen zu schädigen und eine Strahlenreaktion in den direkt getroffenen Zellen zu induzieren. Vorherige Studien zeigen, dass auch nicht direkt der Strahlung ausgesetzte Zellen Strahleneffekte aufweisen. Möglicherweise werden diese Effekte mittels Gap-Junctions, löslicher Faktoren oder extrazellulärer Vesikel kommuniziert. Insbesondere der Einfluss von Exosomen, einer Klasse kleiner extrazellulärer Vesikel, wird derzeit intensiv erforscht. Exosomen sind endosomal sezernierte Vesikel, die aus einer Doppellipidmembran bestehen und Proteine, RNA sowie DNA-Fragmente in Empfängerzellen transportieren. Neueste Studien legen nahe, dass die exosomale Kommunikation eine wichtige Rolle bei der zellulären Stressantwort spielt. Darüber hinaus induzieren Exosomen bei vielen Krebsarten die Onkogenese und fördern die Tumorprogression sowie Metastasierung.

In dieser Doktorarbeit wurde daher untersucht, ob ionisierende Strahlung die exosomale Kommunikation von Kopf- und Halstumoren beeinflusst. Hierbei wurde der Effekt von Strahlung auf die Exosomenfreisetzung und -aufnahme, die exosomale Zusammensetzung und die Auswirkungen auf phänotypische Veränderungen sowie intrazelluläre Signalkaskaden von Empfängerzellen untersucht.

Die in dieser Arbeit beschriebenen Ergebnisse zeigen, dass die exosomale Zell-Zell-Kommunikation eine Komponente der Strahlenantwort von Kopf- und Halstumoren darstellt. Ionisierende Strahlung erhöht die Exosomensekretion sowie -aufnahme und induziert eine modifizierte exosomale Proteinbeladung. Darüber hinaus erhöhen Exosomen aus bestrahlten Zellen das Überleben, die DNA-DSB-Reparatur, die zelluläre Migration und chemotaxisinduzierte Motilität von Empfängerzellen, wodurch ein strahlungsresistenter und invasiver Phänotyp gefördert wird. Interessanterweise induziert Strahlung und der anschließende Exosomentransfer zwischen verschiedenen Zelltypen ebenfalls Veränderungen des zellulären Phänotyps. Diese zelltypunspezifische Kommunikation deutet auf eine hochkomplexe Interaktion zwischen den heterogenen Tumor- und Nicht-Tumorzellpopulationen im bestrahlten Gewebe hin.

Die erhaltenen Daten zeigen weiterhin, dass Exosomen aus bestrahlten Zellen nach endozytotischer Aufnahme den intrazellulären AKT-Signalweg in Empfängerzellen aktivieren. Zusammen mit den bioinformatischen Analysen, welche die deregulierten exosomalen Proteine den Migrationsprozessen und dem AKT-Signalweg zuordnen, deutet dieses Ergebnis darauf hin, dass die exosomalen Komponenten innerhalb der Empfängerzelle funktional sind. Des Weiteren wird die Bedeutung des AKT-Signalwegs für die Aktivierung der strahlen- und exosomenvermittelten Migration durch einen zusätzlich hinzugefügten AKT-Inhibitor (5 µM Afuresertib) nachgewiesen. Die Behandlung von Exosomen mit RNase zeigt, dass neben den Proteinen auch exosomale Nukleinsäuren den zellulären Phänotyp vermitteln und so beispielsweise die DNA-Reparatur beschleunigen.

Abschließend lässt sich zusammenfassen, dass Exosomen biologisch aktive Komponenten in der Strahlenantwort von Kopf- und Halstumoren darstellen. Die neuen Erkenntnisse aus dieser Arbeit zeigen, dass Kopf- und Halstumor-Exosomen eine schützende und tumorfördernde Botschaft an Empfängerkrebszellen senden. Infolgedessen können Exosomen die Strahlenresistenz und die Ausbildung von Metastasen während der Strahlentherapie von Kopf- und Halstumorpatienten fördern und sind daher ein attraktives Ziel, um Strategien der Strahlentherapie zu verbessern.

1 Introduction

1.1 Exosomes

Intercellular communication is an essential process for multicellular organisms and affects biological processes such as cell growth, morphogenesis and differentiation. The crosstalk between direct, adjacent or distal cells is organized by a number of molecular processes. Cell junctions, adhesion contacts, hormones, growth factors, cytokines and extracellular vesicles are all important mediators for the cellular information exchange[1].

Extracellular vesicles are categorized in distinct subgroups according to size, composition and cellular origin. The larger class vesicles, microvesicles, are generated by direct budding from the plasma membrane. A heterogenic-sized population of apoptotic bodies is produced from cells undergoing cell death by apoptosis[2-4]. Exosomes, the smallest subclass of extracellular vesicles, are secreted via endocytosis (Figure 1). They are present in the extracellular space and circulate in body fluids, such as blood, saliva and urine[5-7]. Their existence was discovered more than 30 years ago, when Harding et al. observed a complex vesicle secretion process in maturing blood reticulocytes[8,9]. In 1987 Rose Johnstone termed the nanometer-sized (40 - 150 nm) secreted vesicles ‘exosomes’[10].

1.1.1 Exosome composition and biogenesis

The bilayer lipid membrane of exosomes which is enriched in cholesterol, glycosphingolipids, sphingomyelin and phosphatidylserine encapsulates different classes of RNA, intravesicular or transmembrane proteins as well as DNA fragments[11-13]. Exosomes carry for example mRNA (messenger RNA), small RNA like miRNA (microRNA), long non-coding RNA, tRNA (transfer RNA) and circular RNA[14-18]. Several subsets of protein classes are highly enriched within exosomes, including the tetraspanins CD9 and CD81[19], the endosome-associated proteins Alix and TSG101[20,21] and the heat shock proteins HSP60, HSP70 and HSP90[22].

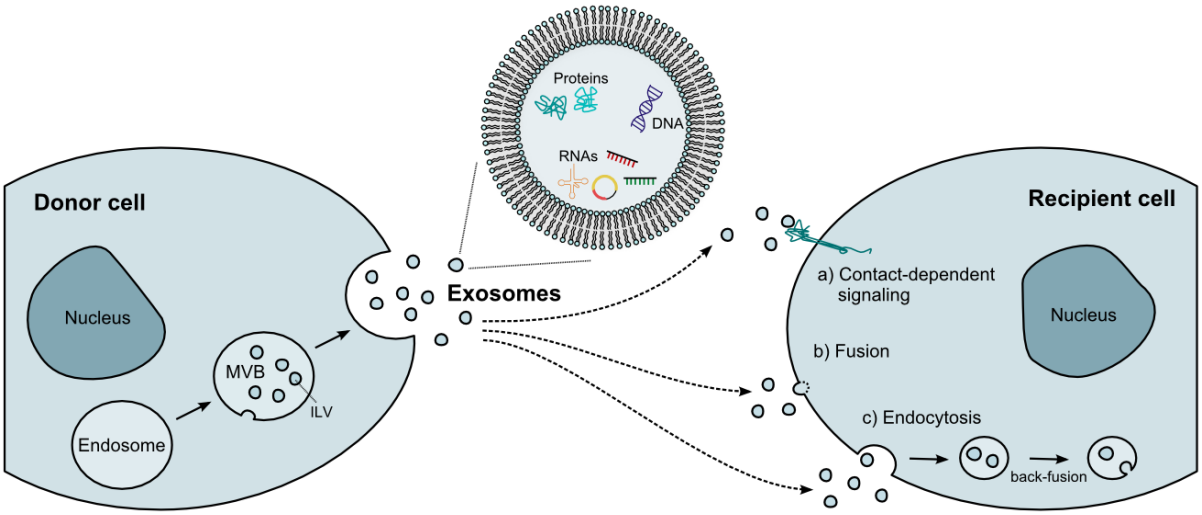

Figure 1: Exosome biogenesis, composition and uptake mechanisms. Multivesicular bodies (MVBs) are generated by inward budding of endosomes. To release exosomes MVBs fuse with the plasma membrane. Exosomes consist of a bilayer lipid membrane, incorporated proteins and nucleic acids. Exosomes interact with recipient cells either via surface-interaction, fusion or endocytosis.

For exosome biogenesis early endosomes mature to multivesicular bodies (MVBs) by inward budding and formation of intraluminal vesicles (ILVs)[23]. ILVs can be generated via different mechanisms. The Endosomal Sorting Complex Required for Transport (ESCRT) is the best characterized transport pathway[12,24], but there are also lipid- and tetraspanin-mediated mechanisms described[19,25-27]. On the basis of different molecular machineries that regulate exosome biogenesis, it was suggested that exosome sub-types may co-exist[28]. The MVBs can then either degrade their content lysosomally or release exosomes into the extracellular space by fusion with the plasma membrane[12]. Vesicles with a high cholesterol content are preferentially released to the extracellular space[29].

The exosomal cargo does not reflect the cellular content and components may be selected for export rather than being randomly incorporated[30-34]. Active and passive sorting mechanisms have been described for biomolecule loading into exosomes. Exosomal lipid sorting occurs during ILV formation, to enrich membranes in sphingomyelin and to reduce the level of phosphatidylcholine[27,35]. Proteins and miRNAs can bind selectively to lipid raft-like regions and can be incorporated and sorted via lipid-mediated processes into ILVs[36-38]. Protein sorting into exosomes is also regulated by post-transcriptional protein modifications, such as ubiquitination, sumoylation, phosphorylation and glycosylation[39]. For instance, sorting of

glycoproteins into exosomes is affected by *N*-linked glycosylation, thereby possibly modulating the chaperoned-loading of non-glycosylated proteins like CD81[40]. However, the role of the post-transcriptional modifications is not completely clear, since ubiquitinated and non-ubiquitinated loading has been described[41,42]. Post-transcriptional protein modifications can also regulate the activity of miRNA shuttle proteins. There is evidence that the loading of miRNAs is regulated by the sumoylated ribonucleoprotein hnRNPA2B1 or RNA binding protein SYNCRIP, both recognizing specific sequences in miRNAs, the so-called EXO-motifs[43,44]. Ago2, a protein of the RISC complex, also controls the uptake of miRNAs into exosomes. Changes in the phosphorylation status of Ago2 are induced by the KRAS-ERK-signaling cascade, causing reduced loading of miRNAs into exosomes and their redirection into processing bodies[45]. The exosomal abundance of miRNAs can also be raised by post-transcriptional nucleotide addition. Koppers-Lalic et al. have found miRNA isoforms with a uridylated 3'-end to be enriched in exosomes[46].

1.1.2 Contact-dependent signaling and internalization of exosomes

Once secreted, exosomes are able to confer their message to recipient cells via cell surface binding, membrane fusion or endocytosis[47]. Exosome attachment to cells can be achieved by cellular receptor molecules that specifically recognize exosomal ligands (e.g. ICAM-1) or lipid classes (e.g. phosphatidylserine)[48,49]. This surface binding can induce intracellular signaling cascades[47]. The internalization of exosomes is accomplished by membrane fusion or endocytotic processes such as phagocytosis, macropinocytosis and receptor- or raft-mediated endocytosis[47,50-52]. If the exosomal content is internalized, exosomes function as a vector for the dissemination of genetically encoded messages. In order to release the exosomal cargo into the cytosol of the recipient cell after endocytosis, a back-fusion step of the exosome with the limiting endosomal membrane is the most likely scenario. The back fusion mechanism is not entirely clear; however the endosomal lipid lysobisphosphatidic acid (LBPA) and the protein Alix were identified as essential components of this process[53-55]. As a consequence the released exosomal material including proteins, RNAs and lipids is functional within an acceptor cell[14,56-58].

1.1.3 Biological functions and effects of exosomes

Interacting and absorbed exosomes are able to modulate various signaling pathways, influence biological functions and can confer new phenotypes to recipient cells. Exosomal communication regulates normal physiology, but also pathological states. For instance, exosomes have immunoregulatory potential[59] and can promote angiogenesis[60-62]. Moreover, exosomes promote the proliferation of cancer cells[63], tumor growth and progression[64,65] as well as the propagation of oncogenic potential[57,66,67]. Cancer cell migration, invasion and metastasis are also increased by exosomes[68-71]. Hereby, they can not only affect the cells at the primary tumor site by inducing pro-metastatic epithelial-to-mesenchymal transition (EMT), but they can also act at distant sites to establish a pre-metastatic niche by promoting the vascular permeability and extracellular matrix remodeling[72,73]. Furthermore, tumor exosomes guide and redirect tumor cells to the preferential tumor cell destinations and thereby determine organotropism[74]. In conclusion, exosomes are important modulators of the tumor stroma interactions and create the environment required for successful tumorigenesis and metastasis. Growing evidence supports not only a role in tumor progression, but also in the tumor response.

1.1.4 Exosomes in the stress response

Exosomal communication may be modified upon exposure to different kinds of stressors. Firstly, stress conditions alter the release and uptake rates for exosomes. For example, the cytostatic compound Doxorubicin increases the release of nasopharyngeal carcinoma cell exosomes[75] while an extracellular acidic environment augments both the release and uptake efficiency of melanoma exosomes[50]. Secondly, stress conditions can be reflected by changes in the exosomal cargo[32,75-77] and can also cause changes in vesicle size[78]. For instance, hypoxia increased the release of breast cancer exosomes carrying miR-210[79]. Thirdly, stress-induced composition changes are able to pass the stress signals to recipient cells. Studies in murine mast cells show that exosomes collected under oxidative stress increase the oxidative resistance in recipient mast cells[77]. Moreover, exosomes from hypoxic and heat-stressed breast and oral squamous cell carcinoma induce migration, invasion and the metastatic potential of acceptor cells[78,80]. Extracellular vesicles isolated from heat-shock-treated cells induce DNA damage and apoptosis in non-stressed cells,

while they beneficially increase heat-stress resistance indicated by reduced DNA damage and apoptosis, in heat-shocked cells[78]. Finally, tumor exosomes may counteract cancer treatment by either transferring a drug resistant phenotype to target cells[56,81] or by inhibiting the tumor-reactive antibody binding to therapeutic target structures[82,83]. For example, a study from Wang et al. demonstrated that non-tumorous bone marrow stromal cell-derived exosomes can induce drug resistance in multiple myeloma cells[84]. These studies show that stress-induced vesicle communication affects biological processes and therefore can alter cancer therapy outcome and prognosis.

1.1.5 Exosomes in a clinical context

The discovery that extracellular vesicles are transporting proteins and genomic material which effectively influence tumor progression and therapy response makes them a very interesting source of biomarkers[85,86]. Tumor biomarkers can facilitate diagnosis and prognosis, but can also help to monitor the treatment response or predict a relapse risk. Circulating exosomes are potential easily accessible biomarkers, since they are present in various body fluids, like blood, saliva and urine[5-7]. Due to the great variety of incorporated cargo, exosomes reflect the genetic alterations and expression changes on several biomolecular levels, such as miRNA, protein and DNA[6,7,87]. Further, based on the selective cargo loading during exosomal sorting, selected markers might be enriched, which would otherwise constitute a small intracellular or extracellular proportion.

Based on the mostly tumorigenic action of exosomes (1.1.3), it may be advantageous to prevent exosome production or to even remove them from the blood by hemofiltration as an adjuvant therapy modality[88]. Alternatively, exosomes could be useful tools in anti-tumor vaccination protocols to deliver tumor-derived antigens and to elicit an anti-tumor immune response[89,90]. Engineered exosomes, that target specifically tumor tissues, could be administered with incorporated anti-tumor compounds. These are currently being tested in *in vivo* models[91,92].

1.2 Ionizing radiation

1.2.1 Effects of ionizing radiation on molecules and cells

Ionizing radiation describes those energetic electromagnetic waves or particles that are able to remove an electron from the outer atomic shell of a target atomic nucleus resulting in formation of ionized atoms and molecules. Biologically relevant ionizations may be generated by either direct interaction with a target molecule or indirectly by generating free radicals. As a consequence, ionizing radiation is able to damage cellular structures like DNA, cytosolic proteins, membrane components or organelles. Damages inflicted on the DNA, especially the formation of DNA double strand breaks (DSBs), are the most detrimental risk. The harmed cells induce a DNA damage response (DDR), which results in arrest of the cell cycle, DNA repair or apoptosis[93]. If these lesions are not repaired properly, they can cause mutations and chromosomal aberrations in the progeny population. Thus, ionizing radiation is a health-risk factor that can drive carcinogenesis, but also induce cardiovascular disease, tissue fibrosis, cataract formation, cognitive defects and memory impairment[94].

1.2.2 Non-targeted radiation effects

The radiation-induced bystander effect (RIBE) was initially described following the observations that cells without any direct energy deposition respond to radiation and exhibit signs of the DDR. It was suggested that gap-junctions or soluble factors, such as reactive oxygen species (ROS) or nitric oxide (NO), mediate the RIBE[95,96]. Thus, the radiation-induced signaling from irradiated tissues can influence tumor cell growth at distant, non-exposed sites of an organism (abscopal effect)[97,98].

The rescue effect describes, in contrast to the RIBE, the phenomenon where irradiated cells receive beneficially signals from non-irradiated bystander cells[99]. For instance, Kobayashi et al. showed that non-irradiated fibroblasts accelerate DNA damage repair in distant irradiated lung cancer cells (rescue effect), while they induce DNA damage in non-irradiated distant fibroblasts (RIBE)[100].

While irradiation has clear cytotoxic effects in targeted cells, it can also induce a more proliferative, migratory and radioresistant phenotype in adjacent cells[101-103]. For instance, fibroblasts released more hepatocyte growth factor (HGF) in response to

radiation, which caused a MET (Hepatocyte growth factor receptor)-hyperactivation, promoted invasive growth and protection from radiation-induced apoptosis of tumor cells[104]. This radiation-induced protection includes also the adaptive response (AR), in which low doses are priming cells to subsequent higher radiation doses by inducing a protective response[105-107].

In conclusion, the target area of radiation can be wider than expected, and the consequence of the tissue- and organismic-specific interaction response during radiotherapy is not fully elucidated. The radiation-induced interaction between cells and tissues might mediate radioresistance of tumor patients. The mechanistic action and complexity of intercellular signaling after irradiation, including beneficial and harmful effects on recipient cells, as well as the role of exosomes should be therefore further analyzed.

1.2.3 Clinical radiation oncology

Based on the cell-killing effect of high doses of ionizing radiation, radiotherapy is a highly effective therapy modality and therefore frequently integrated in the treatment scheme of patients with localized solid tumors[108]. Approximately 50 % of all tumor patients receive radiotherapy, either with the direct intent of curing, or in an adjuvant, neoadjuvant or palliative manner[109,110]. Despite of constant technological improvements and modern high-precision radiotherapy techniques, adverse side effects and radiation resistance are still major challenges of radiotherapy. Normal tissue toxicity, including acute endothelial cell killing and delayed radiation-induced fibrosis limit the radiation dose that can be delivered to a tumor[111]. Successful tumor regression whilst preserving essential organ structures and functionalities is the aim of radiotherapy. For this it is required to maximize radiation dose deposition in cancer cells while minimizing the dose to the surrounding healthy tissue. The effectiveness of radiotherapy could be enhanced by either applying specific radiosensitizers to the tumor or radioprotectors to the normal tissue[112]. In addition, it would be of great benefit to assess individual patient radiosensitivity. However, up to now there is no suitable method for the determination of radiosensitivity established.

A further clinical phenotype, the formation of metastasis is a severe treatment complication, and there is evidence that ionizing radiation modulates the tumor cell

migration on local and distant sites[113,114]. In a study from Strong et al. the incidence of distant metastasis was significantly increased in head and neck cancer patients after receiving preoperative radiotherapy[115]. In accordance, radiation induced head and neck cancer cell line migration in a dose-dependent manner[116]. The underlying mechanism of this radiation-induced effect was studied by modulating the activity of the surface receptor EGFR or components of the downstream pathways. The motile phenotype after irradiation was shown to be mediated via intracellular downstream effectors of the AKT- and ERK-pathways. This is in line with the radiation-induced invasion of glioma cells investigated by Park et al. They found AKT-dependent increased expression and secretion of the matrix-degrading enzyme MMP-2 after irradiation of tumor cells[117].

1.3 Squamous head and neck carcinoma

1.3.1 Epidemiology, risk factors and therapy

Squamous head and neck carcinoma (HNSCC) is the sixth most common cancer worldwide with 529,000 new cases per year[118]. Risk factors for these epithelial malignancies of the oral and nasal cavity, paranasal sinuses, pharynx or larynx are alcohol and tobacco consumption as well as HPV- (human papillomavirus) or EBV- (Epstein-Barr virus) infections[119-121]. Radiotherapy, often in combination with surgery, chemo-, or immunotherapy, is applied for rapid tumor regression and preservation of important organs. The continued development of therapy options over the past decades has increased the overall 5-year relative survival rate from 54.7 % in 1992 - 1996 to 65.9 % in 2002 - 2006[122]. However, the relative increase in the incidence of HPV-positive HNSCC, a subgroup with favorable prognosis, may have led to the observed increased survival[123,124]. Nevertheless, the beneficial effect of radiation in the treatment scheme was verified for advanced tongue, oral cavity and tonsillar carcinoma, suggesting that the continuous evolution of radiotherapy modalities affects the increased survival rate[122].

Despite advances in multimodality therapy, several challenges remain. Radiation resistance, either mediated by proliferative pathway signaling, hypoxia-induced promotion of genetic instability or by resistant cancer stem cell populations, can cause therapy failure[125-127]. Furthermore, local recurrence, second primary tumors as

well as distant metastasis are recognized treatment complications[128,129]. Although patients survive longer, there is no evidence of a simultaneous decrease in the development of distant metastases[130]. The 5-year survival rate of 20 % remains especially low for patients with distant metastases[131]. A better understanding of the molecular pathogenesis of HNSCC, radioresistance and metastasis mechanisms might further improve the long-term survival.

1.3.2 Molecular pathogenesis of HNSCC

Carcinogenesis is a complex and multistep process, whereby the accumulation of multiple genetic alterations results in uncontrolled cell division. The tumorigenic development is driven by gain of oncogene and loss of tumor suppressor gene activity[132]. HNSCC is a heterogeneous disease with multiple molecular abnormalities. Nonetheless, some genes and signaling pathways are frequently affected. The Cancer Genome Altas Network demonstrated that processes of cell cycle, survival, inflammation, angiogenesis, migration, differentiation and oxidative damage are frequently affected by the most common mutations (Figure 2)[133].

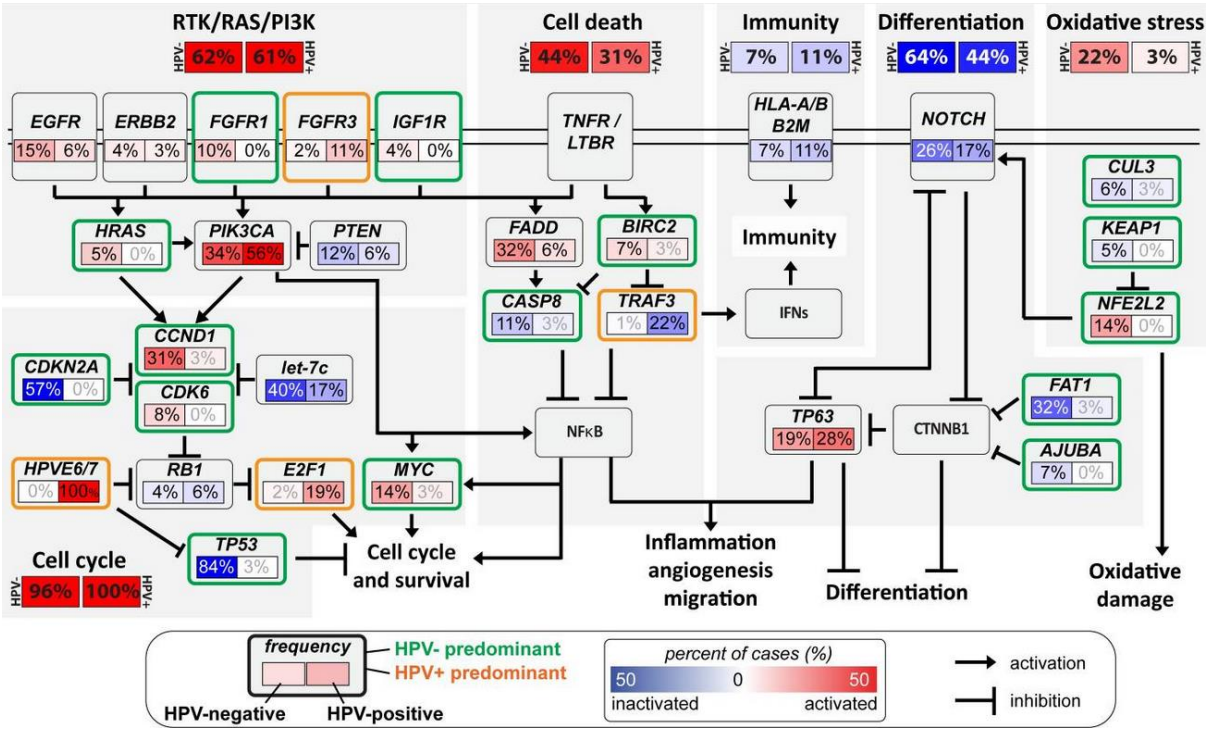

Figure 2: TCGA Deregulation of signaling pathways and transcription factors. Common molecular alterations which affect cell cycle, survival, inflammation, angiogenesis, migration, differentiation and oxidative damage of HPV-negative and -positive HNSCC[133].

Based on the significant proportion of head and neck tumors that are driven by HPV-infections and the difference in molecular dysregulation, pathogenesis and early tumor progression, HPV-positive and -negative tumors form distinct HNSCC subgroups[134]. *TP53*, with approximately 70 - 80 %, is the most frequently mutated tumor suppressor gene in HPV-negative squamous head and neck cancer[133,135]. *CDKN2A*, which is coding for p16-INK4a, is also often inactivated in HPV-negative HNSCC either by deletion, methylation or mutation[136]. Both the somatic mutations in *TP53* and loss of function in *CDKN2A* can cause perturbation of the cell cycle control. HPV-associated tumors harbor mutations in the oncogene *PIK3CA* and amplifications of the cell cycle gene *E2F1*. Moreover, the HPV genome encodes the viral oncoproteins E6 and E7 that inactivate p53 and Rb, respectively[124,133].

The majority of the HNSCC also display mutations in the PI3K-AKT-signaling (1.3.3)[133,137]. Several components of this signaling axis are susceptible to disruption by genetic alterations. Hereby, the *PIK3CA, PIK3CG* and *PTEN* genes were most often found to be affected[135,138]. The oncogenes *EGFR*, *FGFR1* and *FGFR3* are the most frequently amplificated and mutated receptor tyrosine kinases in HNSCC[133]. Interestingly, overexpression of FGFR1 relates with poor overall survival and reduced disease-free survival in HPV-negative HNSCC[139]. Further genes, like *CCND1* (coding for G1/S-specific cyclin-D1), *NOTCH* and *TP63* are often affected, too[124,140,141].

1.3.3 Role of AKT-signaling in HNSCC

The PI3K-AKT-signaling is often deregulated in HNSCC and drives, since it is a critical modulator of cellular processes like cell growth, apoptosis, DNA repair, survival and motility, the oncogenic transformation as well as tumorigenesis[137]. Receptor tyrosine kinases, like EGFR or FGFR1, are activated upon extracellular signals (e.g. ionizing radiation exposure) and mediate the activation via Ras to PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase), which generates increased levels of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) (Figure 3)[142,143]. PIP_3 activates PDK1 and subsequently AKT [144]. Then, mTOR in a complex with Raptor activates p70S6K and rpS6, thus changing gene expression patterns and affecting cellular processes[145,146].

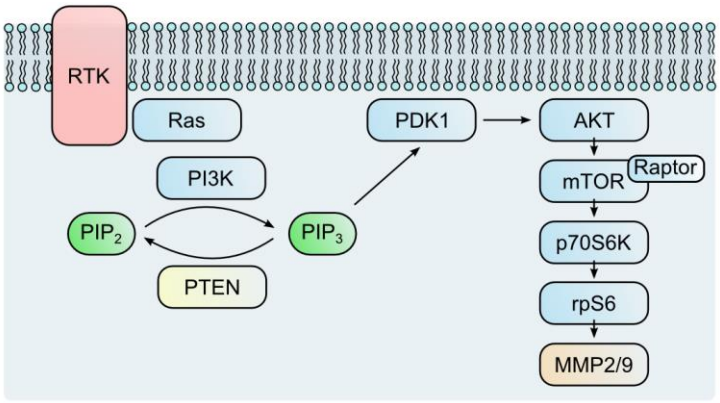

Figure 3: AKT-signaling. Receptor tyrosine kinases mediate the signal via Ras to PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase), resulting in increased levels of phosphatidylinositol-3,4,5-trisphosphate (PIP_3). After cascade activation via PDK1, AKT, mTOR, p70S6K and rpS6, the gene expression of MMP-2 and MMP-9 is modified.

AKT-signaling promotes the tumor cell survival by inhibiting pro-apoptotic protein activity[147] and by degrading p53[148,149]. Moreover, AKT-signaling facilitates DNA-PKcs-dependent DSB repair and thereby mediates radioresistance[150]. In addition, AKT-signaling increases migration and invasion processes by promoting EMT[151-153]. Hereby, AKT regulates the production and activation of MMP-2 and MMP-9, which proteolytically degrade the extracellular matrix barrier, and are therefore critical for the local tumor cell invasion and distant metastasis[154-156]. In line with aberrations in the AKT-pathway their expression is often elevated in HNSCC[157].

The high mutation rate in PI3K-AKT pathway genes, their pivotal role in controlling tumor progression and the availability of specific inhibitors, make AKT a very interesting therapeutic target. Afuresertib is an orally bioavailable, potent and ATP-competitive inhibitor that blocks the kinase activity of AKT by hyperphosphorylation. The clinical efficacy of Afuresertib was proved recently in hematological malignancies, which have also constitutively active PI3K-AKT-signaling[158-160]. Other PI3K-, AKT- and mTOR-inhibitors are also being tested in clinical HNSCC trials[159,161-163]. AKT-inhibitors may abrogate the AKT-mediated radioresistance and migration mechanisms of HNSCC, what makes them interesting tumor radiosensitizers (1.2.3)[126] and metastasis inhibitors[152].

1.3.4 Role of exosomes in HNSCC

The role of exosomes in squamous head and neck carcinoma is not fully understood. First results indicate that exosomes drive head and neck cancer oncogenesis,

progression and metastasis[80,164,165]. Growing evidence supports the concept that HNSCC exosomes suppress the proliferation and activation of T-lymphocytes[166,167]. Further, sera-isolated vesicles from HNSCC patients induce T-cell apoptosis *in vitro*[168]. These results demonstrate the influence of exosomal head and neck cancer communication to effectively impair the anti-tumoral immune response. Moreover, several studies demonstrate that HNSCC exosomes promote the development of a metastatic phenotype. Exosomes from HPV-16-associated oropharyngeal carcinoma induce invasion and EMT of non-tumorigenic epithelial cells[169]. Two studies delineate the role of miRNA transfer in exosome-mediated oral squamous cell carcinoma metastasis. Exosomal miR-1246 promotes cell motility and invasion[170] and hypoxia-induced exosomal delivery of miR-21 results in a pro-metastatic phenotype in recipient oral squamous carcinoma cells[80]. Not only miRNA but also exosomal proteins like MMP-13 and HIF1α are suggested inducers of HNSCC invasion and EMT[171,172].

1.4 Working hypothesis

Ionizing radiation not only damages targeted cells, but also induces a radiation response in non-targeted cells that did not receive any radiation exposure. Intercellular communication via gap-junctions, soluble factors or extracellular vesicles might be responsible for the observed non-targeted cell response. The impact of the nanometer-sized, extracellular exosomes is studied intensively at the moment. Current literature demonstrates that exosomes play a pivotal role in the tumor development as well as cellular stress response.

The here presented work was conducted to investigate the exosome-mediated cell-to-cell communication of head and neck cancer in response to ionizing radiation. Based on recent literature showing altered release and uptake efficiency, modified cargo content and changes in the functionality of exosomes after diverse stress conditions (1.1.4), this doctoral thesis addresses the hypothesis that these parameters are also modulated in HNSCC by ionizing radiation.

For this purpose, the size, morphology and concentration of exosomes isolated from irradiated and non-irradiated donor cells were determined (1.5.2-1.5.3). The uptake of exosomes by irradiated and non-irradiated recipient cells was investigated after transfer of labeled exosomes (1.5.3). To examine the exosomal composition, the

proteome of exosomes isolated from irradiated and non-irradiated cells was analyzed. Subsequently, bioinformatics prediction tools were used to assign the identified and deregulated proteins to biological processes and pathways (1.5.4).

To test the hypothesis that radiation changes the exosome-mediated induction of intracellular signaling pathways and adaption of biological phenotypes, exosomes isolated from irradiated and non-irradiated cells were transferred to recipient cells. For a detailed understanding of the role of exosomes in the radiation response, the DNA repair efficiency, proliferation, clonogenic survival, migration, chemotaxis-induced motility and MMP-activity were tested. The cell type specificity of exosomes was determined by cross-transfer of exosomes between different cell types (1.5.5). Overall, these studies have been designed to test the functional role of tumor-exosomes in response to therapeutic radiation exposure.

1.5 Analysis of exosomal communication in HNSCC after ionizing radiation

1.5.1 Isolation of exosomes

A multitude of isolation methodologies have been established, and are currently in use for the preparation of intact and biologically active exosomes.

The differential ultracentrifugation protocol is the most often applied separation procedure, and is based on the application of a series of centrifugation steps with increasing gravitational force[173,174]. The larger size classes of vesicles, microvesicles and apoptotic bodies can sediment at 10.000 - 20.000 g, while the smaller exosomes pellet only at high speed centrifugation (100.000 g). This differential ultracentrifugation protocol generates moderate exosome yields. However, extravesicular protein aggregates, lipoprotein particles, and other contaminants may be captured by co-sedimentation[175]. Sucrose density gradients or sucrose cushions can reduce the risk of co-sedimentation. Here, exosomes with a density between 1.1 - 1.19 g/ml float up and are separated from vesicles and aggregates with higher densities[175]. This isolation protocol is very laborious and requires extensive methodological skills. Furthermore, studies demonstrate that long centrifugation times, up to 90 hours, are required to achieve a complete density-dependent sorting[176].

Exosome isolation protocols using filtration methods or size-exclusion chromatography are the basis for size-selective vesicle separation. For example, ultrafiltration membranes with a pore size of 0.22 µm will pass only vesicles with a smaller diameter. Although filtration methods are less laborious than centrifugation, aggregates of smaller sized particles are co-separated and contaminate the exosomal suspension[177]. This risk of co-separation can be reduced by size-exclusion chromatography (SEC) and fractionation of the flow through. Columns packed with heteroporous material, which builds up pores and tunnels, allow a slower traverse for smaller molecules, since they occupy the narrow niches for a longer period. The use of gravity flow ensures that the vesicle structure and integrity is not disturbed[178]. The protein contamination is reduced in comparison to non-gradient differential ultracentrifugation protocols, albeit a lower yield is usually obtained[179].

Commercial available polymeric precipitation kits, such as EXOQuick, take advantage of the differential solubility of cellular components. They contain polyethylene glycol (PEG), a substance which was previously successfully used for isolation of virus and other small particles[177]. The drawback of this procedure with little hands-on time is the co-isolation of non-vesicular contaminants, including lipoproteins and a contamination of the exosomal suspension with the precipitation solution.

The methods for affinity-based capture of exosomes use either antibodies against surface proteins including CD63, CD81, CD9, Alix or EpCAM, or use lectins that bind specific saccharide residues on the exosome surface. The highly specific peptide- and carbohydrate-binding proteins are covalently fused to magnetic or agarose beads, plates, chromatographic matrices or microfluidic devices[177,180]. The method has a high selective potential, albeit other extracellular vesicles may be co-isolated, or exosome subpopulations that lack the selected marker molecule might get lost[175]. Hence, the power of affinity-based capture would be increased if multiple exosomal structures would be targeted simultaneously.

Microfluidic devices under development make use of different technological principals: Exosomes can be captured via affinity-based approaches (e.g. immune-chips), sieved and filtered by pressure or electrophoresis (e.g. nanoporous membranes) or trapped on porous structures (e.g. nanowire-on-micropillars).

Especially hybrid approaches where a separation is based on size as well as immunological features are of great interest and might be particularly useful for diagnostic applications[181].

An overview of the most commonly applied isolation methods revealed that most publications apply differential ultracentrifugation. The use of density gradient and cushion protocols has declined, while commercially available methods increase in popularity[174]. Overall, the deficits in purity, efficiency, reproducibility and exosome yield, plus the required specialist technical equipment and technological expertise of the different isolation protocols, have impeded standardization in methodology. Based on the great isolation variety and the missing standardization, the comparability and reproducibility of different studies are limited. Indeed, the isolation methods affect protein and RNA yield, the exosomal cargo profile and the potential biological action[174,182,183].

For this doctoral thesis exosomes were isolated from conditioned media of the head and neck cancer cell lines BHY and FaDu by differential ultracentrifugation with a prior pore filtration step. Thereto, cells were seeded, the medium replaced after 24 hours with exosome-free medium and subsequently irradiated with 0, 3, 6 and 9 Gy (Gray) from a ^{137}Caesium source (γ-rays, dose rate 0.45 Gy per min). The conditioned medium was collected 24 or 48 hours after irradiation, centrifuged at 10.000 g to remove cells as well as cellular debris and filtered (pore size 0.22 µm) to separate larger vesicle classes. In the next step, the filtrate was centrifuged with 100.000 g for 75 minutes at 4°C. After discarding the supernatant, the pellet was resuspended in PBS and centrifuged for an additional 75 minutes at 100.000 g and 4°C. Finally, the exosome pellet was resuspended in fresh PBS and stored at -20°C.

In summary, improved isolation methods are required to generate exosome preperations for subsequent analysis. It is crucial that researchers report experimental details, use standardized protocols and adequately address the quality of exosomal preparations[184,185].

1.5.2 Characterization and validation of exosomes

For quality control and initial characterization, exosomes should be visualized with electron microscopy, quantified with optical or current-based devices and analyzed

for the presence of specific exosomal marker proteins. Electron microscopy provides direct evidence for the presence of vesicular structures and the size of the isolated vesicles. However, transmission electron microscopy (TEM) requires dehydration and fixation of the sample, causing shrinkage and an artificial cup-shaped morphology[173]. In contrast, cryo-electron microscopy analyzes frozen samples without affecting the vesicular structure[186]. Further approaches to determine vesicle size are nanoparticle tracking analysis (NTA, e.g. NanoSight), dynamic light scattering (DLS, e.g. Nano ZS) as well as resistive pulse sensing (RPS, e.g. qNano). The NTA is an optical particle tracking method which records the scattered laser beam to calculate the single particle velocity on the basis of Brownian motion[187]. Dynamic light scattering, also analyzing fluctuations in the intensity of scattered light, however, is restricted to monodisperse suspensions. The NTA visualizes vesicles on an individual basis, and can therefore analyze polydispersed structures between 50 - 1.000 nm[4]. Resistive pulse sensing uses electric potential changes instead of optical recording. Transient reductions of the current are measured during the traversal of individual vesicles through a defined membrane nanopore. In line with the NTA, the RPS can analyze polydisperse suspensions[187]. Further, bead-coupled exosome marker antigens or fluorescent exosomal labels can be determined by flow cytometry, when they exceed the flow cytometer detection threshold of approximately 200 nm[188]. Conventional immunoblotting, ELISA (enzyme-linked immunosorbent assay) or mass spectrometry can also be used to verify the presence of exosomal marker proteins and the absence of typically non-vesicular proteins (1.5.4).

To validate the quality of the exosome preparations of the presented thesis, the proteins Alix, TSG101, CD63, HSP70 and Calnexin were immunoblotted in exosomal and cellular lysates. The total exosomal protein composition was identified by mass spectrometry and compared with databases listing commonly detected exosomal proteins. Furthermore, to identify vesicle structure and size of exosomes isolated from irradiated and non-irradiated cells, nanoparticle tracking analysis (NanoSight) and transmission electron microscopy were performed.

1.5.3 Characterization of exosome release and uptake

Stress situations including exposure to ionizing radiation affect the exosome release and uptake rates[50,75]. The effect of radiation on head and neck cancer exosome signaling kinetics has not been studied.

Optical particle tracking methods, electric resistive pulse sensing and flow cytometry (1.5.1) can be used to quantify exosomes in suspension, and thus, determine exosomal release rates. Although NTA and RPS yield concentration values as number of particles/ml, the absolute quantification between the single methods differs substantially[189]. Based on these technical limitations, relative quantification should be used rather than absolute amounts. In this doctoral thesis the nanoparticle tracking analysis (NanoSight) was used to determine the relative change in exosome release upon exposure to ionizing radiation.

The uptake of exosomes by recipient cells can be studied with different fluorescent labeling technologies. Exosomal membranes (e.g. PKH67, PKH26), total protein (e.g. CFSE (carboxyfluorescein succinimidyl diacetate ester)) or total RNA (e.g. SYTO® RNASelect™Green) content as well as specific exosomal cargo molecules can be labeled[190]. The exosomal uptake can be monitored by fluorescence microscopy and quantified with flow cytometry. Furthermore, active uptake processes can be blocked by lowering the temperature to 4°C or by the addition of endocytosis-inhibitors including Dynasore and Cytochalasin D[52,191,192]. In the here presented work, exosomes were labeled with the lipophilic PKH67 and added for 3, 6, 8, 10 and 24 hours to recipient cells and monitored using flow cytometry and fluorescence microscopy. To study the effect of ionizing radiation on exosomal uptake, the recipient cells were also pre-irradiated with 0, 2 and 4 Gy. In a further approach the transfer of exosomal protein was examined by CFSE-labelling of exosomal proteins. The membrane permeable CFSE is metabolized into a fluorescent form by the activity of exosomal esterases and is covalently coupled via its succinimidyl group to the amino ends of exosomal proteins[193]. CFSE-positive exosomes were transferred to the same type and cross-transferred to different cell types to study cell type specificity of the exosomal protein uptake. To block the uptake of CFSE-positive exosomes, cells were pre-treated with 25 µM Dynasore for 1 hour.

1.5.4 Analysis of the protein composition of exosomes isolated from irradiated donor cells

Detailed knowledge about the exosomal proteome can help to clarify exosome biogenesis processes, uptake and cellular effects as well as support the discovery of candidate exosomal tumor-biomarkers[194,195]. The characterization of the exosomal protein cargo can be accomplished by immunoblot, ELISA or via mass spectrometry. In comparison to RNA or DNA studies, protein-investigating approaches lack the opportunity to amplify the starting material, and therefore require high amounts of exosomes. However, the high performance proteomics approach, which is an unbiased method to qualitatively identify proteins and to quantify their expression, requires only moderate exosomal protein amounts. For sample preparation of the exosomal proteins, in-gel digestion, in-solution digestion or filter-aided sample preparation, followed by mass spectrometry analysis (e.g. LC-MS/MS), can be applied[196]. Hereby, global and targeted as well as stable isotope labeling (e.g. SILAC (stable isotope labeling by/with amino acids in cell culture)) or label-free quantification methods have been developed[195]. Advances in spectrometry-based proteomic tools, like hardware, workflows and informatics have improved the depth of exosomal proteome coverage, and thus, the analysis of the molecular protein composition of exosomes[197].

To analyze the protein composition of exosomes isolated from irradiated and non-irradiated HNSCC cells we used the label-free quantitative analysis. The workflow including filter-aided sample preparation, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis on Orbitrap and database searches, was performed as previously described[198]. *In silico* localization, pathway and biological process analysis of the identified and deregulated proteins was performed using ExoCarta[199], STRING: functional protein association networks[200] and the Reactome 5.1.0 application[201] in the Cytoscape 3.2.1 software[202].

1.5.5 Changes of intracellular signaling pathways and biological effects in exosome recipient cells

The investigation of exosomal function is a leading study topic in the exosome research field[174]. By passing specific messages from cell to cell, exosomes have the potential to affect the fate of recipient cells. A better understanding of the underlying

mechanisms is therefore of great interest. Different concentrations of exosomes can be transferred to recipient cells and their effect investigated after defined exposure times[63,64]. Conversely, their effect can be tested after removal of exosomes from the conditioned medium and transfer of the exosome-free supernatant[203]. Treatment with RNase, DNase, proteinase, chemical detergents or thermal denaturation can all inactivate exosomes[204-206]. These controls can further help to understand what carriers of the exosomal messages are directed to recipient cells. Exosomal uptake pathways can also be blocked. For example, Dynasore, an endocytosis-inhibitor, impedes the exosomal uptake[207].

In this thesis, exosomes isolated from irradiated and non-irradiated cells were transferred onto recipient head and neck cancer cells to study their effect on signaling pathway induction as well as different biological endpoints. A possible influence on the AKT-signaling was tested by determining the cellular phosphorylation level of the AKT downstream targets mTOR and rpS6 after exosome transfer. To explore the mechanism of exosome-mediated pathway induction, the phosphorylation level of the AKT target mTOR was analyzed after endocytosis-inhibition (25 µM Dynasore) and subsequent exosome transfer. Apart from signal transduction experiments, various protocols investigating the exosomal effects on biological processes have been established and applied. Here, cell proliferation after exosome transfer was studied with the Presto Blue™ Cell Viability Reagent and by determining the cellular plating efficiency. In addition, the exosomal influence on the cell survival after exposure to different irradiation doses was investigated with the clonogenic survival assay. To assess information about the DNA repair capacity, 53BP1-foci-detection was used for quantification of DNA double strand breaks after irradiation and subsequent exosome transfer. Control exosomes were pre-treated with RNase A (5 µg/µl or 400 µg/µl) or a detergent-peptidase-mixture (0.2% Triton X-100/Trypsin, 2:1). A gap-closure assay, based on wound healing migration, was applied to determine the migratory potential of cells after exosome treatment. To analyze the role of the AKT-signalling in the exosome-mediated migration, the AKT-inhibitor Afuresertib (5 µM) was added simultaneously with the exosomes to cells in the migration assay. The chemotaxis-induced motility after exosome transfer was monitored in the xCELLigence® Real-Time Cell Analyser

(RTCA) DP System. Zymography, an SDS- (Sodium dodecyl sulfate) and gelatin-based electrophoretic technique was used to measure the gelatinase activity of the supernatant collected from exosome- and Afuresertib-treated (5 µM) cells. Beyond that, cell type specificity was tested for the transfer of DNA double strand break repair efficiency and the cellular migratory potential. Hereby, the exosomal crosstalk between different head and neck cancer cell lines, fibroblasts and endothelial cells was analyzed.

1.5.6 Workflow

The experimental approach of this thesis is explained in paragraphs 1.5.1-1.5.5 above and graphically summarized in Figure 4. Exosome isolation, characterization of exosomal cargo, release and uptake efficiency were performed after irradiation of head and neck cancer cells with 0, 3, 6 and 9 Gy in a ^{137}Caesium source. Furthermore, exosome transfer experiments were performed to access information about the functional role of exosomes in recipient cells during the radiation response.

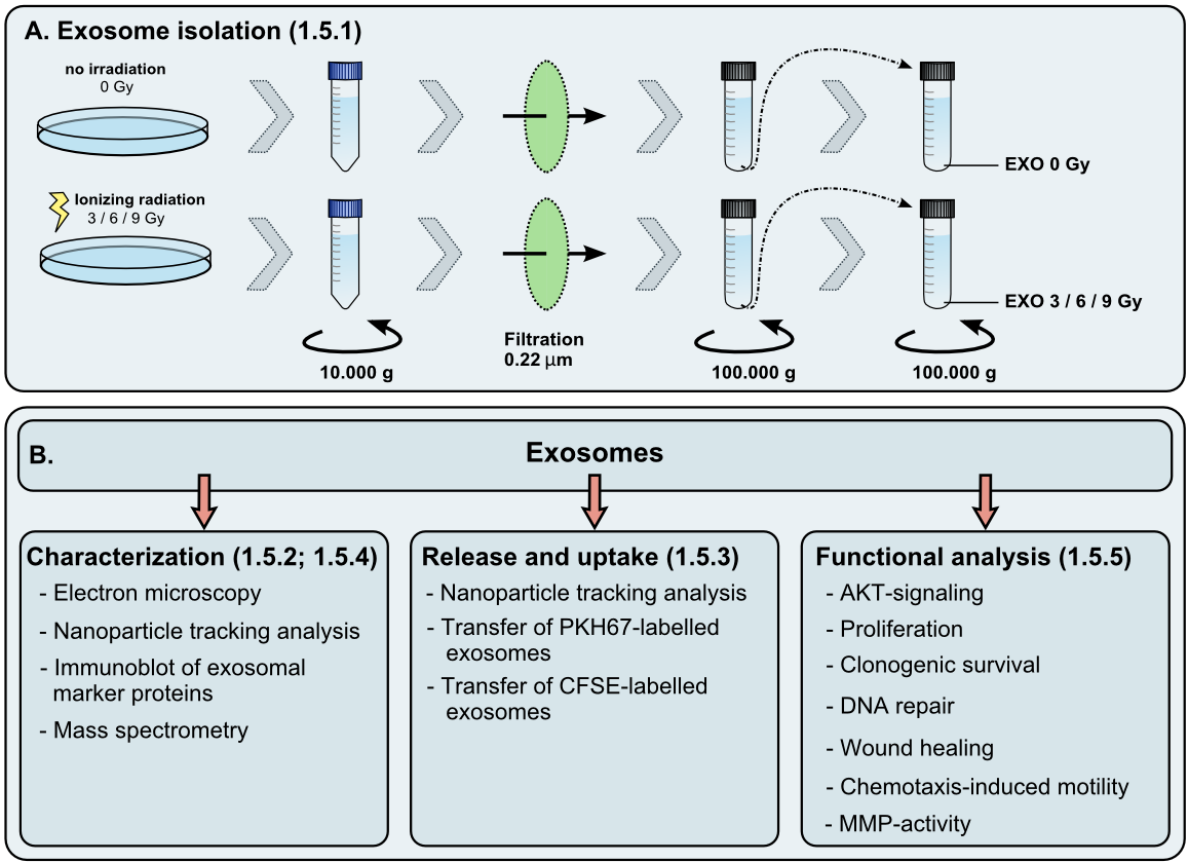

Figure 4: Workflow. (A) Exosome isolation protocol. **(B)** Overview of the exosome-investigating experimental approaches, including exosomal characterization, exosomal release and uptake efficiency as well as biological function of exosomes.

2 Results and Discussion

2.1 Exosomes derived from squamous head and neck cancer promote cell survival after ionizing radiation

2.1.1 Aim and summary of the study

Besides damaging target cells, ionizing radiation also induces non-targeted radiation effects in cells that where not exposed to irradiation directly. It was suggested that this radiation response is mediated via gap-junctions, soluble factors or extracellular vesicles (1.2.2). However, the role of exosomal signaling in the radiation response is not understood. Previous work demonstrated that exosomes are essential components in the tumor development, progression and response during stress situations (1.1.3-1.1.4). The aim of this study was to analyze exosomes in the radiation response of head and neck cancer cells. Specifically, the effect of irradiation on the exosomal release and uptake rates and the impact of exosome transfer on proliferation, survival and DNA repair capacity of recipient cells were studied.

The results of this study show that exosomal communication plays a role in the radiation response of squamous head and neck cancer cells. Both exosome release and uptake by a HNSCC cell line are increased after ionizing radiation. Furthermore, although exosomes received from irradiated and non-irradiated cells equally increased the cellular proliferation, exosomes from irradiated cells had a more potent effect on the survival of exosome-recipient cells. This is in line with the observed enhancement in DNA DSB repair efficiency. Treatment of exosomes with either RNase or a detergent-peptidase-mixture abrogates the accelerated DNA repair.

Thus, exosomes released by head and neck cancer cells are bioactive, and may contribute to the radiation stress response as well as to the development of radioresistance in head and neck cancer patients. In summary, a better understanding of the underlying mechanisms of exosomes and an abrogation of their protective action might improve strategies for radiation therapy.

2.1.2 Contribution

For this study, I performed the cell cultivation, irradiations and exosome isolations. Moreover, I conducted the immunoblotting, NanoSight measurements, uptake experiments and the investigation of the DNA DSB repair kinetics. Klaudia Winkler supported the immunoblotting and uptake experiments by providing her technical assistance. Furthermore, Klaudia Winkler and Dr. Simone Moertl conducted the proliferation, plating efficiency and clonogenic survival experiments following my study design. Carsten Peters, PhD, performed the electron microscopic experiments on samples I prepared. In addition, I performed the data analysis and interpretation. Moreover, I designed all the figures, illustrations and tables. I wrote the manuscript with the help of Dr. Simone Moertl. The co-authors Dr. Simone Moertl, Prof. Dr. Michael J. Atkinson, Dr. Ramesh Yentrapalli and Theresa Heider contributed to the scientific discussion and data interpretation.

The Western Blot of exosomal marker proteins and some results of the DNA DSB repair kinetic experiments were performed as part of my master's thesis (Masterarbeit: Charakterisierung extrazellulärer microRNAs nach Bestrahlung von HNSCC Zelllinien, Lisa Mutschelknaus, 2014, Ludwig-Maximilians-Universität München).

2.1.3 Publication

The data was presented in the following original research paper on March 23th 2016 in 'PloS ONE'.

Exosomes Derived from Squamous Head and Neck Cancer Promote Cell Survival after Ionizing Radiation

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RESEARCH ARTICLE

Exosomes Derived from Squamous Head and Neck Cancer Promote Cell Survival after Ionizing Radiation

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DSB, double-strand break; EXO, exosomes; HNSCC, head and neck squamous cell carcinoma.

Abstract

Exosomes are nanometer-sized extracellular vesicles that are believed to function as intercellular communicators. Here, we report that exosomes are able to modify the radiation response of the head and neck cancer cell lines BHY and FaDu. Exosomes were isolated from the conditioned medium of irradiated as well as non-irradiated head and neck cancer cells by serial centrifugation. Quantification using NanoSight technology indicated an increased exosome release from irradiated compared to non-irradiated cells 24 hours after treatment. To test whether the released exosomes influence the radiation response of other cells the exosomes were transferred to non-irradiated and irradiated recipient cells. We found an enhanced uptake of exosomes isolated from both irradiated and non-irradiated cells by irradiated recipient cells compared to non-irradiated recipient cells. Functional analyses by exosome transfer indicated that all exosomes (from non-irradiated and irradiated donor cells) increase the proliferation of non-irradiated recipient cells and the survival of irradiated recipient cells. The survival-promoting effects are more pronounced when exosomes isolated from irradiated compared to non-irradiated donor cells are transferred. A possible mechanism for the increased survival after irradiation could be the increase in DNA double-strand break repair monitored at 6, 8 and 10 h after the transfer of exosomes isolated from irradiated cells. This is abrogated by the destabilization of the exosomes. Our results demonstrate that radiation influences both the abundance and action of exosomes on recipient cells. Exosomes transmit prosurvival effects by promoting the proliferation and radioresistance of head and neck cancer cells. Taken together, this study indicates a functional role of exosomes in the response of tumor cells to radiation exposure within a therapeutic dose range and encourages that exosomes are useful objects of study for a better understanding of tumor radiation response.

1 Introduction

Exosomes are a subclass of extracellular microvesicles that are secreted by most cell types, including tumor cells. They are endocytic in origin and released into the extracellular environment through fusion of cytosolic multivesicular bodies with the plasma membrane. Exosome cargo includes a wide range of proteins, mRNAs, microRNAs and long non-coding RNAs [1–4]. Functional studies reveal that exosomes act as extracellular communicators by delivering their content to a target cell via membrane fusion, or alternatively by endocytosis [5]. In 2007 Valadi et al. demonstrated that exosomes are able to shuttle RNA between cells. The transfer of murine mast cell exosomes to human mast cells results in the translation of murine mRNA, proving that the delivered RNA molecules are functional in the recipient cells [3].

Absorbed exosomes are able to modify biological functions of the recipient cells, where they may confer a new phenotype, such as metastasis [6], angiogenesis [7] and migration [8]. The exosomal composition of the extracellular milieu is modified by cellular stressors, leading to changed, mostly protective effects upon recipient cells. Thus exosomes derived from cells exposed to oxidative stress provide resistance against oxidative stress to non-exposed recipient cells [9]. In breast cancer cell lines, hypoxia also increases the release of exosomes carrying increased amounts of miR-210. This enhances survival and invasion of recipient cells [10]. In the context of ionizing radiation exosomes derived from irradiated glioma cells enhance the migration of recipient glioma cells [11]. Exosomes may thus influence communication of radiation effects between non-targeted and targeted cells (bystander-like signaling), such as genomic instability [12–14].

Squamous cell carcinomas are common malignancies of the head and neck region. Radiochemotherapy or radiotherapy is the most common therapy for HNSCC (head and neck squamous cell carcinoma) patients with locally advanced and unresectable tumors [15]. However, therapy resistance and tumor recurrence pose a major challenge and their mechanisms are not well understood. Since exosomes are emerging players in drug resistance we aim to evaluate whether exosomes could affect the radiation response of head and neck squamous carcinoma cells [16–19]. For this purpose we determined the impact of ionizing radiation within a moderate dose range on exosome release and uptake in HNSCC. In order to analyze a putative functional role of exosomes we added exosomes isolated from differentially irradiated donor cells, and analyzed resulting effects on proliferation, survival and DNA repair of recipient HNSCC after a treatment with ionizing radiation.

2 Materials and Methods

2.1 Cell culture and irradiation

Head and neck cancer cell lines BHY (DSMZ no.: ACC 404) and FaDu (ATCC®HTB43™) were incubated at 37°C and a relative air humidity of 95%. BHY cells were cultivated in high Glucose DMEM culture medium (Dulbecco's modified Eagle's medium, Gibco) plus 10% fetal calf serum (Bio&SELL), 2 mM L-Glutamine and sodium pyruvate at 10% CO_2. FaDu cells were maintained in low Glucose DMEM (GE Healthcare) supplemented with 10% fetal calf serum, 2 mM L-Glutamine and 25 mM HEPES at 5% CO_2. Cell line identities were validated by sequencing of nine different loci: D5S818, D13S317, D7S820, D16S539, VWA, TH01, AM, TPOX, CSF1PO (performed by Eurofins Genomics, S1 and S2 Tables). A mycoplasma screening revealed negative results.

Cells were irradiated with γ-rays emitted by a ^{137}caesium source at the irradiation facility HWM-D2000 (Wälischmiller Engineering) with a dose rate of 1 Gy per 2.04 minutes.

2.2 Isolation of exosomes

For the isolation of exosomes the protocol of Théry et al. was adapted [20] (Fig 1A). 5×10^5 cells were seeded per 10-cm petri dish, 72 hours later the medium was replaced by 8 ml exosome-free medium and cells were irradiated over a moderate dose range of 0–9 Gy. Exosomes isolated from non-irradiated cells received the abbreviation EXO 0 Gy, while exosomes from irradiated cells were named EXO 3 Gy, EXO 6 and EXO 9 Gy. Exosome isolation was conducted from the conditioned medium collected 24 and 48 hours after irradiation. To eliminate detached cells, dead cells as well as cellular fragments, the cell culture supernatant was centrifuged with 10,000 g for 30 minutes and afterwards passed through a filter with a pore size of 0.22 μm. An ultracentrifugation step with 100,000 g enabled the sedimentation of the exosomes (75 minutes, 4°C). The supernatant was discarded and the exosomal pellet was resuspended in 2 ml PBS. After repetition of another ultracentrifugation step (100,000 g) the supernatant was discarded and the exosomes were resuspended in PBS. Exosomal preparations were stored at -20°C. Exosome donor cells were harvested 24 and 48 hours after irradiation using a cell scraper. After washing the cellular pellet twice with PBS, the pellet was frozen at -20°C. For the preparation of exosome-free medium, bovine exosomes were removed from fetal calf serum by centrifugation at 100,000 g for 14 hours.

2.3 Electron microscopy of exosomes

Undiluted sample (isolated from 3 ml conditioned medium) was absorbed onto glow discharged carbon coated grids (G2400C from Plano) for 2 minutes. The solution was blotted of and negatively stained with 4% ammonium molybdate (Sigma-Aldrich) solution for 30 seconds. Micrographs were recorded with a Jeol JEM 100CX electron microscope at 100 kV onto Kodak SO163 film. Negatives were digitized with a Hasselblad Flextight X5 scanner at 3000 dpi, resulting in a pixel size of 0.25 nm/px. For visualization images were binned to 1 nm/px.

2.4 Exosome quantification and determination of exosomal size-distribution

Exosome amount and size distribution was analyzed by using the NanoSight LM10 (Malvern) microscope. Exosome preparations (isolated from 5 ml conditioned medium) were diluted 1:100 to 1:2000 with H_2O to achieve 15 to 50 particles per frame for tracking. Samples were each analyzed three times for 30 seconds.

2.5 Exosome uptake

Exosomes (isolated from 15 ml conditioned medium) were stained with the green fluorescent dye PKH67 (MINI67-1KT, SIGMA-Aldrich Chemie). For this purpose 50 μl of exosome solution were resuspended in 250 μl of the diluent C plus 1.5 μl of the dye (1 mM). After 10 minutes of incubation at room temperature excessive dye was removed by using Exosome Spin Columns (Invitrogen) according to the manufacturer's protocol. As control an equal amount of dye in diluent C plus 50 μl of PBS was processed similar to exosomes (exosome negative control, -EXO).

To measure the uptake of exosomes 50,000 cells in 200 μl medium were seeded in 48 well plates. After 24 hours equal amounts of PKH67-stained exosomes were added to irradiated and non-irradiated recipient cells. After an additional 3, 6, 8, 10 and 24 hours cells were washed three times with PBS, trypsinized and resuspended in 500 μl of PBS. Uptake was measured on a FACSCAN LSRII (Becton-Dickinson, excitation = 490 nm, emission = 502 nm). For fluorescence microscopy cells were washed three times with PBS fixed with 4% paraformaldehyde

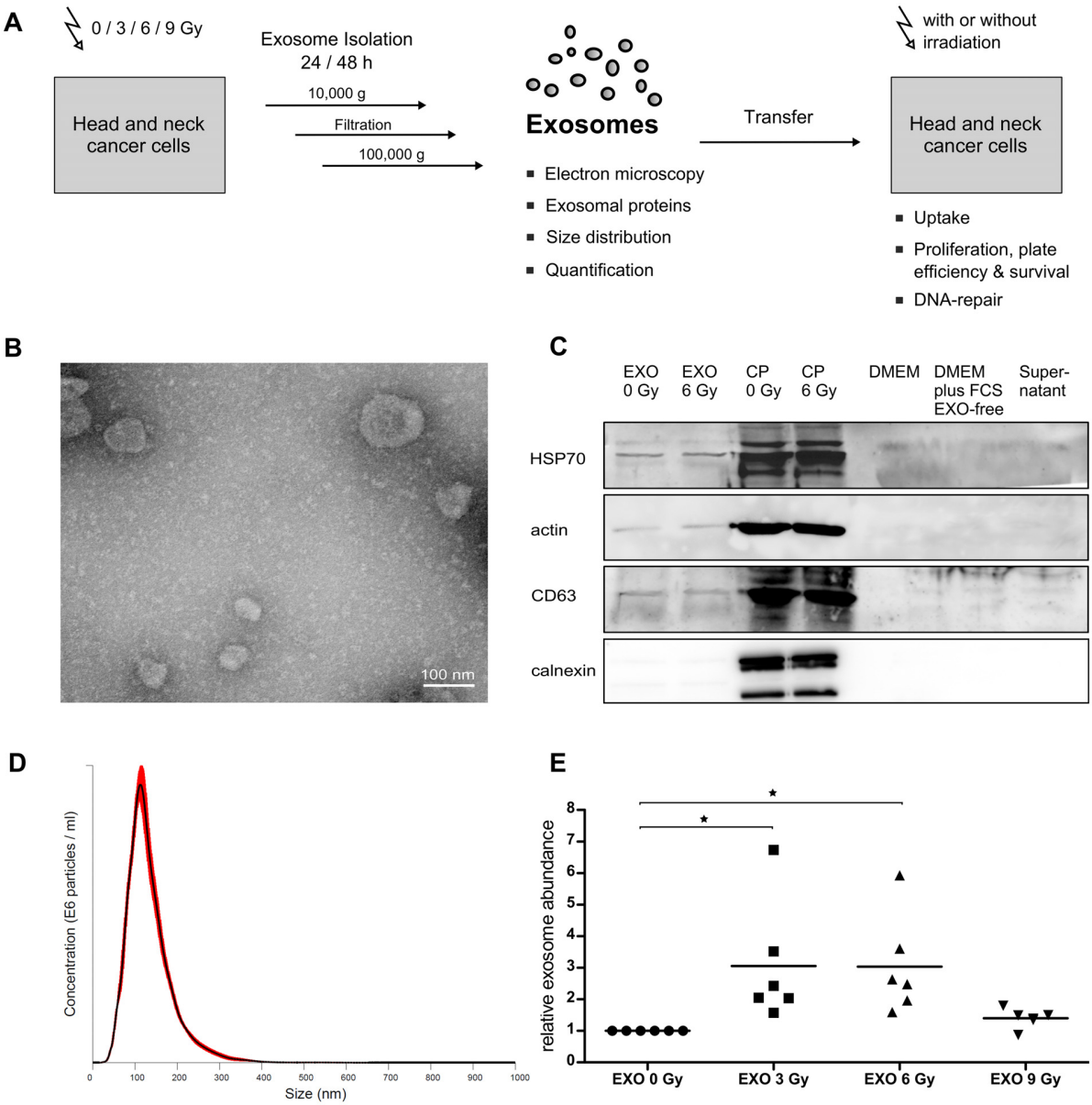

Fig 1. Characterization of isolated exosomes. (A) Scheme of exosome analysis. **(B)** Transmission electron micrograph showing exosomes isolated from the cell culture supernatant of 3 Gy-irradiated BHY cells [scale bar: 100 nm]. **(C)** Representative immunoblot of HSP70, actin, CD63 and calnexin performed with exosome lysates (EXO) and cell lysates (CP) harvested 24 hours after irradiation. DMEM medium, DMEM medium supplemented with exosome-depleted fetal calf serum as well as supernatant after ultracentrifugation were loaded as controls. **(D)** Size distribution of exosomes from non-irradiated BHY cells measured with NanoSight technology. **(E)** Relative exosome abundance of BHY exosomes isolated 24 hours after irradiation with 0, 3, 6 and 9 Gy [n = 6, p-value < 0.05].

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washed again with PBS and covered with Vectashield® including Hoechst 33342 for nuclei staining. Pictures were taken with the fluorescence microscope BZ-9000 from Keyence.

2.6 Incubation of recipient cells with exosomes

To determine the biological activity of exosomes (proliferation, survival and DSB repair) we incubated the recipient cells with exosomes isolated from identical numbers of donor cells. The

exosomes were recovered into volumes to give a three-fold concentration of exosomes compared to the native conditions.

2.7 Proliferation and clonogenic survival after transfer of exosomes

The effect of exosomes on the proliferation was determined with the Presto Blue™ Cell Viability Reagent Protocol (Life Technologies). 500 or 1500 cells per well were seeded into 96 well plates in 100 μl exosome-free medium. After 24 hours exosomes (isolated from 300 μl conditioned medium) were added and the cells were incubated for another 72 hours. For the measurement of cell proliferation 10 μl Presto Blue reagent were added per well, incubated for 40 min at 37°C and fluorescence was determined (Excitation 560 nm; Emission: 590 nm) in a plate reader (Tecan).

For survival determination, a clonogenic survival assay was performed. Cells were seeded in 12 well plates and sham treated or irradiated with 1, 2, 3, 6 and 10 Gy. Immediately afterwards, exosomes (from 2.5 ml conditioned medium) were transferred on the cells which were then incubated for 5 days to allow colony formation from single cells. Subsequently cells were washed twice with PBS, fixed with 100% ethanol (30 minutes) and finally stained with Giemsa solution (Boehringer Ingelheim, 1:20 in PBS, 30 minutes). Excessive dye was removed and colonies with more than 30 cells were counted.

2.8 Detection of DNA double-strand breaks after transfer of exosomes

1,000 to 6,000 cells were seeded in 96 well plates. After reaching a confluence of 50–70% the medium was replaced by 100 μl of exosome-free medium, the cells were immediately irradiated with 2 Gy and exosomes isolated from 300 μl conditioned medium were added. After an incubation of 1, 6, 8 or 10 hours at 37°C the number of DNA DSBs was determined by 53BP1 staining. A fixation step with 4% paraformaldehyde was followed by a permeabilization with 0.2% Triton X-100. Subsequently the cells were blocked with PBS + (1% bovine serum albumin, 0.15% glycine) for 60 minutes and incubated overnight with the primary antibody 53BP1 (dilution 1:500, NB100-305, Novus Biologicals) at 4°C. On the following day the cells were incubated with the secondary antibodies goat anti-rabbit Alexa-488 (dilution 1:200, A-11034, Life Technologies) and sheep anti-mouse Cy-3 (dilution 1:500, 016-160-084, Jackson Lab) for 1 hour. Nuclei were stained with Hoechst 33342 (SIGMA-Aldrich Chemie) and the cells were covered with Vectashield® Mounting Medium (Linaris). Analysis was performed with the fluorescence microscope Biorevo BZ-9000 (Keyence). For all experimental conditions the exposure times were maintained and the foci number of 60 cells per condition was determined.

2.9 Validation of exosomal stability

To test the stability, exosomes were incubated for 30 minutes at 37°C either with RNase A from Qiagen (5 μg/μl or 400 μg/μl) or a detergent-peptidase-mixture (0.2% Triton X-100/Trypsin, 2:1). Then the exosomes were used in DNA repair assays as described above.

2.10 Protein analysis

Cells were lysed in lysis buffer II (25 mM Tris pH 7.5, 120 mM NaCl, 1% Triton X-100, 1% PSMF, 1 mM NOV, 1 mM Leupeptin) for 1 hour on ice. After centrifugation the protein concentration of the collected supernatants was determined by applying the BCA-assay using bovine serum albumin as standard (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific).

Western blot analysis was accomplished according to standard procedures using 10 μg of cellular protein and a volume of 12 μl exosome lysate corresponding to the exosome amount in

30 ml conditioned medium for SDS polyacrylamide gel electrophoresis. Separated proteins were blotted on nitrocellulose membranes and incubated with primary antibodies directed against CD63 (sc15363, SantaCruz), HSP70 (MA3-007, Affinity Bioreagents), actin (SAB1305567, SIGMA-Aldrich Chemie) and calnexin (sc11397, SantaCruz). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (sc2004 and sc2005, SantaCruz) were used to detect antigen antibody binding via chemoluminescence (Amersham ECL detection kit, GE Healthcare).

2.11 Statistical analysis

Data represent the mean of independent, biological replicates ± standard deviation (SD). Significance of n-fold changes was calculated by using the paired t-test. To compare means of three or more variables the two-sided ANOVA was applied. For all statistical analysis $p < 0.05$ was considered statistically significant and $p < 0.01$ and $p < 0.001$ was deemed highly significant.

3 Results

3.1 Radiation increases exosome release from head and neck cancer cells

Exosomes released by the head and neck tumor cell line BHY were isolated by differential ultra-centrifugation. To validate the isolation method exosomes were visualized by transmission electron microscopy. The representative micrograph showed round, cup-shaped structures with a diameter of 30–100 nm (Fig 1B). For further verification of the exosome identity the exosomal marker proteins HSP70, actin and CD63 were detected by western blot in BHY exosomes as well as in lysates of BHY cells. No detectable proteins were present in unused culture medium, in unused medium supplemented with exosome-depleted fetal calf serum or in the supernatant of conditioned medium after ultracentrifugation. The absence of calnexin in the exosome lysates demonstrates that exosome preparations were not contaminated with cell membranes derived from apoptotic bodies or dead cells (Fig 1C). Furthermore the size distribution and the number of isolated exosomes were quantified in six independent preparations for each treatment using NanoSight technology. This approach confirmed a homogenous exosome preparation with an average size of 111–124 nm (n = 6) for the exosomes isolated from either irradiated or non-irradiated cells (Fig 1D). The NanoSight measurement also showed an increase in the number of exosomes recovered from irradiated (EXO 3 Gy, EXO 6 Gy) compared to non-irradiated (EXO 0 Gy) cells 24 hours after irradiation (Fig 1E).

3.2 Radiation increases the uptake of exosomes by recipient cells

We compared uptake kinetics of exosomes isolated from irradiated and non-irradiated donor cells as well as their uptake by irradiated and non-irradiated recipient cells. Therefore cells were co-cultured with PKH67-labeled exosomes and exosome uptake was followed by fluorescence microscopy and flow cytometry.

Fluorescence microscopy revealed a time dependent uptake of exosomes. After 3 hours clusters of labelled exosomes began to accumulate along cell membranes. Increasing numbers of exosomes attached over time and caused diffuse cytoplasm labelling, which implied an internalization and breakup of exosomes (Fig 2A).

We also quantified the exosome uptake by flow cytometry. The obtained results confirmed our previous microscopy observations and showed that the uptake of exosomes was time dependent (Fig 2B) and linear with the added number of exosomes (Fig 2C). The effect of

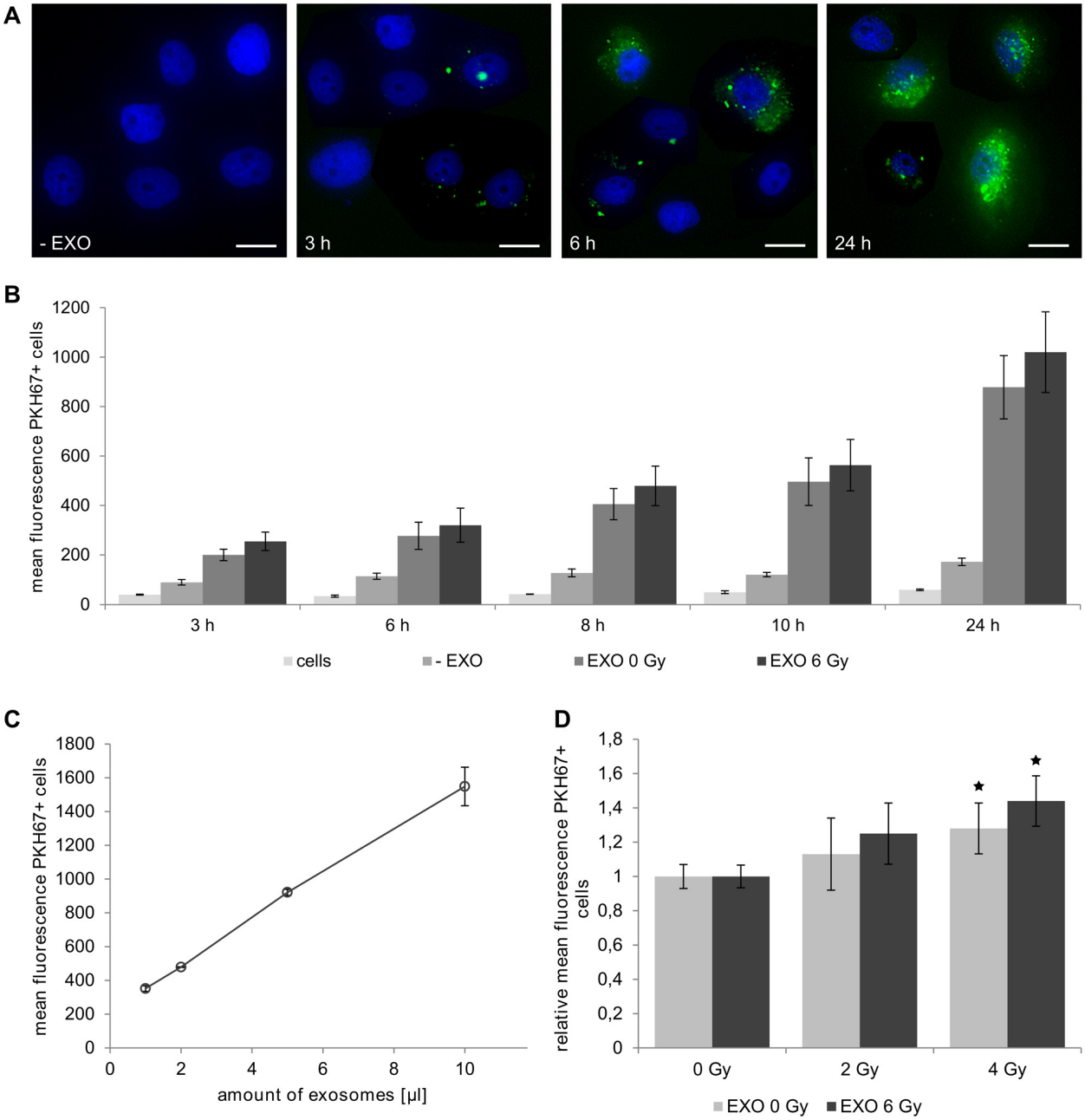

Fig 2. Uptake of exosomes by recipient cells. PKH67-labeled exosomes isolated from irradiated and non-irradiated BHY cells were co-cultivated with BHY cells. **(A)** Representative fluorescence microscopy images for exosome uptake after 3, 6 and 24 hours incubation. Exosomes were stained in green and nuclei were stained blue with Hoechst 33342. **(B)** Uptake of exosomes isolated from 6 Gy-irradiated (EXO 6 Gy) and non-irradiated BHY cells (EXO 0 Gy) after 3, 6, 8, 10 and 24 hours incubation. Mean fluorescence of untreated cells and cells after incubation with stained exosomes or an exosome-negative control (-EXO) is shown (n = 3). **(C)** Dependency of exosomal uptake was determined after 24 hours by using a serial dilution of an exosome preparation. **(D)** Uptake of labeled exosomes by 0, 2 and 4 Gy-irradiated recipient cells after 24 hours. In all experiments a minimum of 10,000 cells were analyzed for each sample [$n \geq 3$, ± SD, p-value < 0.05].

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radiation on the uptake of exosomes by recipient cells was investigated by comparing the uptake kinetics of exosomes isolated from non-irradiated cells to those exosomes isolated from irradiated cells. Fig 2B shows that there was no significant difference in the kinetics between the uptake of exosomes derived from irradiated or non-irradiated donor cells. There was, however, a dose-dependent increase of the uptake of exosomes by irradiated recipient cells compared to that by non-irradiated cells. Thus, exosomal uptake was significantly increased 1.3-fold for exosomes derived from non-irradiated cells and 1.4-fold for exosomes from irradiated donor cells if they were incubated for 24 hours with irradiated recipient cells (4 Gy) compared to uptake by non-irradiated recipient cells (Fig 2D). Taken together these results showed that exosome uptake by recipient cells was time and concentration dependent and that irradiation of recipient cells increased their ability to take up exosomes.

3.3 Exosomes from either non-irradiated or irradiated cells increase survival of recipient cells

We were interested whether exosomes from irradiated cells exhibit the same biological effects in the recipient cells as exosomes from non-irradiated cells. To address this question we added exosomes isolated from donor cells irradiated with 0, 3, 6 and 9 Gy to non-irradiated recipient cells and measured cell proliferation. BHY cells treated with exosomes showed greater proliferation than cells cultivated without exosomes (Fig 3A). Accordingly the plating efficiency in the colony formation assay is greater for cells grown with exosomes than for cells grown without exosomes (Fig 3B). However, no significant difference was detected between treatments with exosomes isolated from 0, 3, 6 or 9 Gy-irradiated cells (Fig 3A).

Next the influence of exosomes on the radiation sensitivity of BHY cells was analyzed. Cells were incubated with exosomes, then irradiated with doses of up to 10 Gy and incubated for 5 days. Subsequently, the clonogenic survival was determined. In accordance with the observed proliferation-stimulating effect of exosomes on non-irradiated recipient cells (Fig 3A and 3B) the survival of irradiated recipient cells was increased by the addition of exosomes (Fig 3C and S3 Table). Here, the exosomes isolated from cells irradiated with 6 Gy induced a greater level of radiation resistance than exosomes from non-irradiated cells (Fig 3B). These results suggest that exosomes from BHY cells generally support proliferation and radiation resistance.

3.4 Exosomes affect rates of DNA double-strand break repair

Since Dutta et al. showed that exosomes released from breast cancer cells can alter the phosphorylation status of DNA damage repair proteins [21], we analyzed the rate of DNA double-strand break (DSB) repair in irradiated recipient cells to elucidate the mechanism for the increased survival of cells after addition of exosomes. Exosomes from irradiated and non-irradiated BHY cells were transferred to irradiated BHY cells (2 Gy) and the number of DNA DSB foci was analyzed after 1 and 6 hours. Quantification of DNA DSB repair foci 1 hour after radiation exposure revealed no difference in the number of induced foci between control cells and cells incubated with exosomes either from non-irradiated or from irradiated donor cells (Fig 4A). Six hours after treatment we found a decreased number of repair foci in BHY cells incubated with exosomes isolated 24 hours after irradiation of BHY cells when compared to cells incubated with exosomes from non-irradiated BHY cells, suggesting a quicker rate of repair (Fig 4B and 4C). Similar effects were seen after 6 hours for exosomes isolated 48 hours after irradiation (Fig 4C). Also the analysis of the distribution of foci numbers per cells after incubation with exosomes reflected the increased repair in cells treated with exosomes from irradiated donor cells. Especially the number of cells with high foci number (> 12) is decreased after incubation with EXO 6 Gy (S1A Fig). Moreover the observed effects were also present 8 and 10

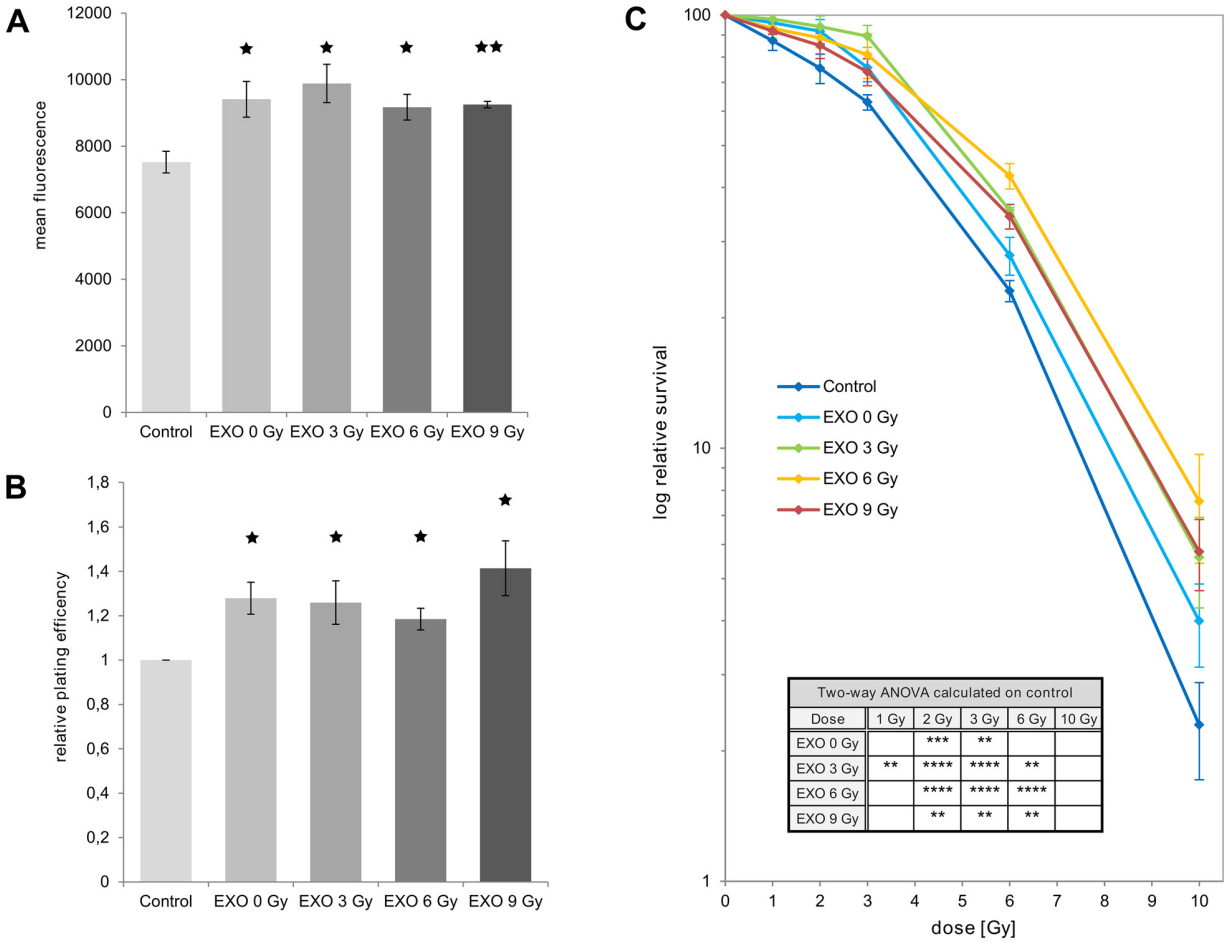

Fig 3. Exosomes affect proliferation, colony formation and clonogenic survival. (A) Proliferation of cells cultivated for 3 days in medium containing exosomes isolated from irradiated or non-irradiated cells. As a control an equal amount of PBS without exosomes was added to the recipient cells. **(B)** Plating efficiency of cells cultivated for 5 days in medium containing exosomes isolated from irradiated or non-irradiated cells. As a control an equal amount of PBS without exosomes was added to the recipient cells. **(C)** Clonogenic survival of BHY cells co-cultivated with exosomes isolated from irradiated or non-irradiated cells and control cells (BHY + PBS) were incubated for 5 days after irradiation with the indicated doses [n = 3, ± SD, p-value: * if $p < 0.05$, ** if $p < 0.01$ and **** if $p < 0.0001$].

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hours after irradiation (S1B Fig). If the cells were pre-incubated for 24 hours with the exosomes, then irradiated with 2 Gy and fixed after 6 hours, the quicker repair induced by exosomes from irradiated donor cells was still observable (S1B Fig). An addition of exosomes from non-irradiated BHY cells appeared to slightly increase the foci number in comparison to the control (PBS) in the recipient cells 6 hours after irradiation (Fig 4B and 4C). This effect was not present after the pre-incubation of cells with exosomes and subsequent irradiation (S1C Fig).

Exosome-stimulated DNA repair was confirmed using a second head and neck cancer cell line FaDu. Again the incubation of FaDu recipient cells with exosomes isolated from irradiated FaDu cells decreased the amount of DNA repair foci (Fig 4D). To test the cell type specificity of exosome-induced effects we added exosomes isolated from BHY cells to irradiated FaDu cells. Exosomes from BHY cells are able to execute similar radioprotective effects on FaDu cells (Fig 4E).

Finally we destabilized exosomes through high concentration RNase A treatment or by adding a detergent-peptidase-mixture. Destabilized 0 Gy and 6 Gy exosomes were unable to

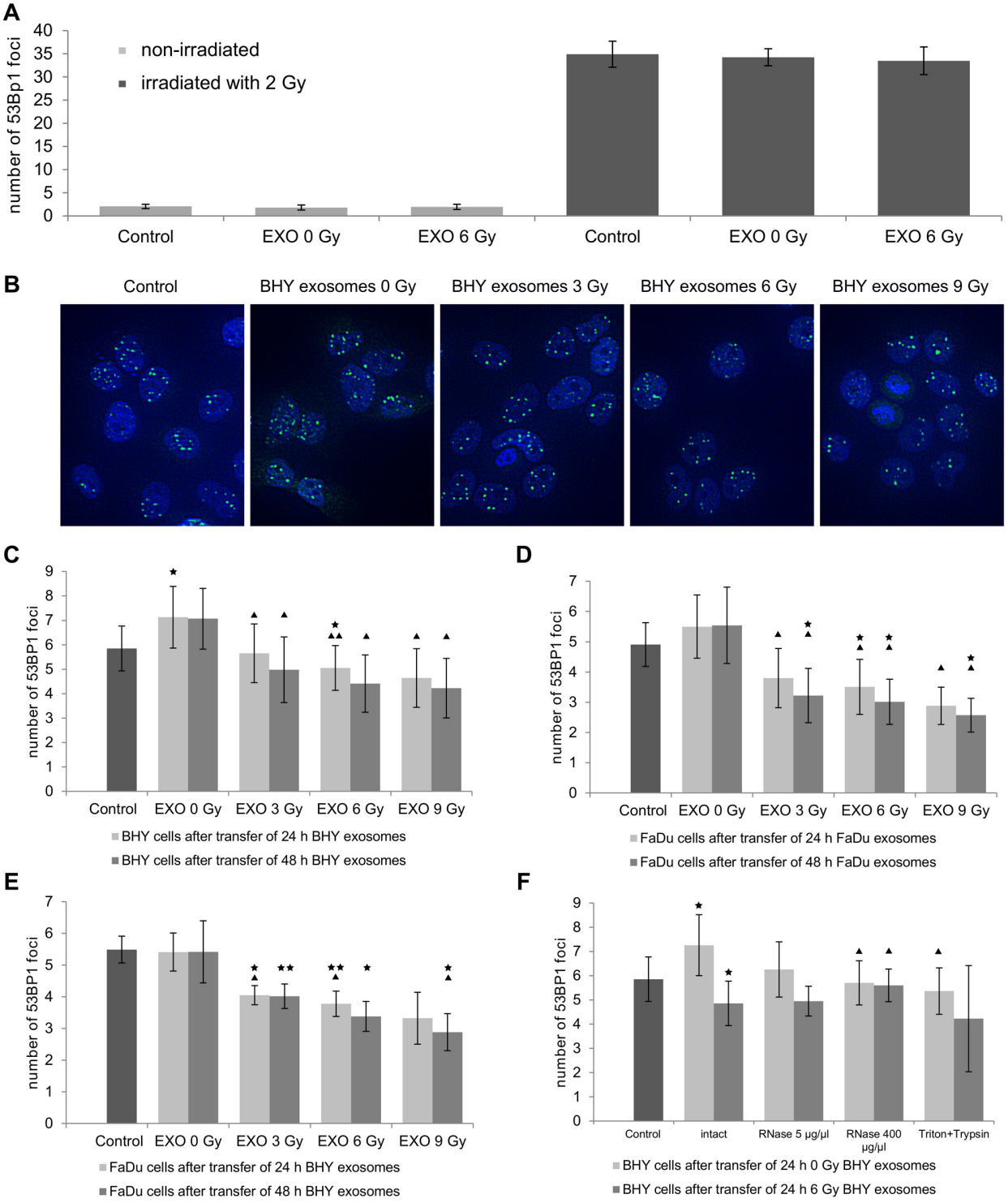

Fig 4. Exosomes modulate the repair of DNA DSBs in irradiated recipient cells. (A) Number of 53BP1 foci in BHY cells 1 hour after irradiation with 0 and 2 Gy and transfer of BHY exosomes isolated 24 hours after irradiation with 0 and 6 Gy [n = 5]. **(B)** Representative images of 53BP1 foci in BHY cells 6 hours after 2 Gy and transfer of BHY exosomes isolated 24 hours after irradiation with 0, 3, 6 or 9 Gy (53BP1 foci green, nuclei blue). **(C)** Number of 53BP1 foci in BHY cells 6 hours after 2 Gy and transfer of BHY exosomes isolated 24 and 48 hours after irradiation [n_1 (control; EXO 0 Gy 24 h; EXO 6 Gy 24 h) = 6, n_2 (EXO 0 Gy 48 h; EXO 3 Gy; EXO 6 Gy 48 h; EXO 9 Gy) = 3]. **(D)** Number of 53BP1 foci in FaDu cells 6 hours after 2 Gy and transfer of FaDu exosomes [n = 3]. **(E)** Number of 53BP1 foci in FaDu cells 6 hours after 2 Gy and transfer of BHY exosomes [n = 3]. **(F)** Number of 53BP1 in BHY cells after 2 Gy and

transfer of destabilized BHY exosomes. Exosomes from BHY cells isolated 24 hours after irradiation with 0 and 6 Gy were treated with RNase A or a mixture of Triton and Trypsin [n_1 (control; intact) = 6; n_2 (RNase A 5 μg/μl) = 2; n_3 (RNase A 400 μg/μl; Triton + Trypsin) = 3]. For all experiments the ± SD was shown and p-values calculated on control were considered to be significant if * $p < 0.05$ and highly significant * * if $p < 0.01$, while ▲ $p < 0.05$ and ▲▲ $p < 0.01$ indicate significant differences to EXO 0 Gy.

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change the number of repair foci in comparison to untreated exosomes indicating a loss of function due to the treatment (Fig 4F). Summarizing these results, exosomes influence the repair of DNA DSBs in a dose dependent, cell type unspecific manner.

4 Discussion

Cell communication via exosomes is able to influence the fate of cells in stress situations [9, 10, 22, 23]. We now show a contribution of exosomes to the increased survival of head and neck cancer cells after irradiation. Exosomes secreted within 24 hours after irradiation have an impact on proliferation, cell survival, and DNA repair efficiency. As a consequence, cell communication via exosomes during anti-tumor radiation may promote resistance of cancer cells and enhance survival of head and neck cancer cells both, in and outside of the radiation field. Therefore, a better understanding of the underlying mechanisms of exosomes in the radiation response will be needed to improve strategies for radiation therapy.

4.1 Exosomes increase survival and proliferation of head and neck squamous carcinoma cells

We show that exosomes influence the fate of irradiated and non-irradiated BHY and FaDu head and neck cancer cells. Exosomes increase the survival of irradiated recipient cells. The prosurvival effects of exosomes from irradiated donor cells were more pronounced than those induced by exosomes from non-irradiated donor cells. In accordance Hazawa et al. showed that the transfer of exosomes from non-irradiated cells to 8 Gy-irradiated mesenchymal stem cells results in increased survival [24].

Correspondingly, exosomes induce proliferation in non-irradiated recipient cells. This effect is independent of radiation-treatment of the exosome donor cells. In the recent literature the effects of exosomes on proliferation are discussed controversially. Similar to our results, exosomes derived from bladder cancer cells, chronic myeloid leukemia cells, or mast cells increase the proliferation of recipient cells after exosome transfer [8, 25–27]. However, Jella et al. showed reduced viability of keratinocytes after incubation in exosome-containing culture medium [14].

4.2 Exosomes affect the DNA double-strand break repair after ionizing radiation in head and neck squamous carcinoma cells

We hypothesized that exosomes may promote survival by triggering DNA repair as it was shown that phosphorylation of critical DNA repair proteins is influenced by exosomes [21]. Our results showed that DNA repair was not influenced by exosomes at an early time point after irradiation (1 h), while increased DNA repair was found after incubation with exosomes from irradiated donor cells at later time points (6–10 h). As the increased DNA repair was equally detected for a 6 h incubation at which only a limited number of exosomes is associated to the cells and after a pre-incubation with exosomes we assume that a small amount of exosomes is sufficient to induce the observed effects. Different aspects of the impact of exosomes on the DNA repair were analyzed in two recent studies. One showed that an increased number of DNA repair foci was observed after transfer of exosomes from non-irradiated breast cancer

cells to normal human primary mammary epithelial cells [21]. Using the comet-assay, Al-Mayah et al. on the other hand showed that exosomes increase the DNA damage of breast epithelial cancer cells [12]. However, both studies focus on the effect of exosomes on non-stressed cells while we provide data about the effects on radiation-stressed cells.

Several studies show that different cell lines exchange cellular components via exosomes suggesting that cell communication via exosomes is not cell type specific. Exosomes of colorectal cell lines for example deliver their content to hepatoma and lung cancer cell lines [3, 28]. In line with this, we verify that exosomes from BHY cells induce the same effects in the DSB repair in FaDu cells. This example is further evidence that exosomes are an intercellular communication tool and corroborates their already-suggested broad cell specificity. This is of great relevance for radiation therapy as the communication between irradiated and non-irradiated cells may be an important regulator of therapy outcome.

4.3 Radiation increases exosomal release and uptake in head and neck squamous carcinoma cells

In addition to the analysis of exosomal effects on recipient cells we focused on the exosomal release and uptake in the context of radiation. Irradiation increases the number of exosomes in the cell supernatant, suggesting that radiation augments the overall amount of exosome release. This is in accordance with studies describing radiation-increased exosome release in glioblastoma, prostate cancer and lung cancer cells [11, 29, 30]. Irradiation with the high dose of 9 Gy reduces the exosome release compared to 3 and 6 Gy irradiated cells. Possibly the enhanced damage increased the induction of cell death processes and counteracts the release of exosomes. Irradiation does not change the exosomal size, whereby the isolated exosomes from BHY cells with an average size of 111–124 nm are at the upper size limit.

We confirm the influence of radiation on the uptake of exosomes by using fluorescence-labeled exosomes. Fluorescence microscopy pictures visualized the attachment of clusters of exosomes to the cell membrane at an early time point followed by their internalization and distribution in the cytoplasm at later time points. FACS analysis further demonstrated a dose-dependent increased uptake of exosomes by irradiated recipient cells. We assume that irradiation induces the uptake of exosomes by recipient cells. This finding is in accordance with the increased uptake of exosomes by mesenchymal stem cells and glioblastoma cells upon irradiation through augmented CD29/CD81 complex formation [24]. An increased uptake of exosomes from irradiated donor cells as shown for glioblastoma cells is not detected in this study for head and neck cancer cells [11]. However, we cannot conclude if exosomes have to be internalized or if they induce the observed effects through the association to the cell membranes alone.

4.4 Exosome cargo increases resistance against tumor eliminating therapies

The development of therapy resistance is the limiting factor of cancer treatments. An exosome-conferred increase in drug resistance has been shown for several cell lines and compounds [16–19]. We demonstrate that exosomes from irradiated donor cells also increase radiation resistance and increase DNA repair in head and neck squamous carcinoma cells. A decreased α/β-ratio of the survival curve after transfer of exosomes also suggests an increase in the DNA repair capacity (S3 Table). However, exosomes from non-irradiated donor cells also produced a slight increase in radiation resistance, while they did not accelerate DNA double strand break repair it is obvious that also other pathways beside repair contribute to the increased survival. Basically exosome quantity and exosome cargo may contribute to the observed biological

effects. But as the number of released exosomes and the biological effects do not correlate, we suggest that exosomal effects are mainly caused by a change in exosomal composition or cargo. Several investigations reveal that cellular stress can alter the exosomal RNA composition [10, 11, 22]. According to these findings, and based on the finding that RNase treatment abrogates the effects of exosomes on DNA repair, we suggest that exosomal RNA molecules (either attached to or included into exosomes) may trigger repair processes in recipient cells. This finding for extracellular RNA stands in line with our previous studies which showed that the expression of intracellular microRNA and long non-coding RNA supports survival of irradiated cells [4, 31, 32].

5 Conclusion

We have evaluated the role of exosomes in the response of head and neck cancer cells to radiation. Our results show that exosomes can serve as a communication tool in the acute radiation stress-response and confer protective signals to neighboring cells. We conclude that exosomes transmit prosurvival signals and therefore promote the tumorigenic and radioresistant phenotype of head and neck cancer cells. This study indicates a functional role for exosomes in the response of tumor cells to therapeutic radiation exposure and encourages that exosomes are useful targets to improve therapy strategies.

Supporting Information

S1 Fig. (A) BHY cells were categorized according to the foci number per cell (0–29). For each experiment the foci number of 60 BHY cells was determined 6 hours after irradiation with 2 Gy and transfer of BHY exosomes isolated 24 hours after irradiation with 0 and 6 Gy [n = 3]. **(B)** Relative number of 53BP1 foci in BHY cells 6, 8 and 10 hours after 2 Gy and transfer of BHY exosomes isolated 24 hours after irradiation [n_1 (6 h control; 6 h EXO 0 Gy; 6 h EXO 6 Gy) = 6, n_2 (6 h EXO 3 Gy; 6 h EXO 9 Gy; 8 h; 10 h) = 3, ± SD]. **(C)** BHY cells were pre-incubated with exosomes, irradiated 24 hours later and the number of 53BP1 foci was determined 6 hours after irradiation [n = 3, ± SD]. For all experiments the p-values calculated on control were considered to be significant if * $p < 0.05$ and highly significant ** if $p < 0.01$, while $^{\blacktriangle}$ $p < 0.05$ and $^{\blacktriangle\blacktriangle}$ $p < 0.01$ indicate significant differences to EXO 0 Gy.
(TIFF)

S1 Table. Authentication of BHY cell line. A short tandem repeat profile was obtained by PCR amplification of eight core short tandem repeat loci plus amelogenin for sex determination. Authentication of cells was performed by comparing the results with the online DMSZ Profile Database (www.dmsz.de). In the diagram the best fitting five cell lines of this alignment with the database are depicted. The authentication for BHY matches to 100%.
(XLS)

S2 Table. Authentication of FaDu cell line. A short tandem repeat profile was obtained by PCR amplification of eight core short tandem repeat loci plus amelogenin for sex determination. Authentication of cells was performed by comparing the results with the online DMSZ Profile Database (www.dmsz.de). In the diagram the best fitting five cell lines of this alignment with the database are depicted. For the tested FaDu cells the best fitting database profile was obtained from FaDu cells with a 88.3% match.
(XLS)

S3 Table. Clonogenic survival of BHY cells. Data were plotted on a semi-log scale and fitted to the linear quadratic equation $SF = e^{(-\alpha D-\beta D\hat{}2)}$. Parameters α and β were used to calculate the α/ β ratio, the inactivation dose for 37% survival (D37) and the surviving fraction at a dose

of 2 Gy (SF2).
(XLS)

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Author Contributions

Conceived and designed the experiments: LM SM MA. Performed the experiments: LM CP KW RY TH. Analyzed the data: LM CP SM MA. Contributed reagents/materials/analysis tools: CP SM MA. Wrote the paper: LM CP RY TH MA SM.

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2.2 Radiation alters the cargo of exosomes released from squamous head and neck cancer cells to promote migration of recipient cells

2.2.1 Aim and summary of the study

Recent evidence demonstrated that ionizing radiation induces a motile phenotype in many cancer types (1.2.3). In addition, exosomes promote the invasion capacity and tumor migration to local and distant sites (1.1.3-1.1.4). However, a possible interaction between ionizing radiation and extracellular vesicle-communication in squamous head and neck cancer migration is not documented. This study therefore investigated the effect of ionizing radiation on exosome communication and the subsequent impact on motility processes of recipient cells. Furthermore, the exosomal protein composition changes after radiation were studied.

Exosomes from irradiated head and neck cancer cells promoted the wound healing capacity, chemotaxis-induced migration, MMP-activity and caused the activation of the pro-migratory AKT-signaling pathway in recipient cells. The impact of AKT-signaling on the migration induction was confirmed by AKT-inhibition (5 µM Afuresertib) at the time of exosome transfer. Furthermore, inhibition of endocytosis with 25 µM Dynasore blocked the AKT-pathway induction, and demonstrated that the uptake of the exosomal content via endocytosis affects signaling pathways rather than surface attachment or direct fusion. In accordance, proteomic and bioinformatics analyses assigned the majority of the 75 deregulated exosomal proteins to migration processes and AKT-activation. The results from this study suggest that the radiation-induced exosomal protein alterations caused the phenotypic adaptions and pathway activations.

In conclusion, exosomal communication is an essential part of the head and neck cancer cell-to-cell signaling in response to ionizing radiation exposure. As a consequence, exosomes may act as driver of HNSCC progression during radiotherapy, and therefore represent attractive targets for clinical intervention.

2.2.2 Contribution

For this study, I performed the cell cultivation, irradiations and exosome isolations. Moreover, I conducted the NanoSight analysis, wound healing assays, chemotaxis-induced motility assays, immunoblotting and uptake experiments. Rosemarie Kell

performed the zymography assays under my supervision and assisted, with Klaudia Winkler, in immunoblotting. Vanja Radulović and Dr. Nataša Anastasov provided the stable GFP-transduction of the cell lines. Marcus Vetter developed the program Image Colour Analyser for the analysis of the wound healing assay. Prof. Dr. Stephan M. Huber and Dr. Lena Edalat helped me with the chemotaxis-induced motility assays. The mass spectrometry analysis of exosomes was conducted by Dr. Omid Azimzadeh, Dr. Juliane Merl-Pham and PD Dr. Soile Tapio. I performed the bioinformatics analyses of the identified and deregulated proteins with the help of Theresa Heider and Dr. Omid Azimzadeh. In addition, I designed the study, performed the complete data analysis, conducted the data interpretation and created all the figures, illustrations, videos as well as tables. I wrote the manuscript with the help of Dr. Simone Moertl, Prof. Dr. Michael J. Atkinson and Theresa Heider, who all joined the scientific discussion and data interpretation, too.

2.2.3 Publication

The data was presented in the following original research paper on September 29th 2017 in 'Scientific Reports'.

Radiation alters the cargo of exosomes released from squamous head and neck cancer cells to promote migration of recipient cells

Lisa Mutschelknaus, Omid Azimzadeh, Theresa Heider, Klaudia Winkler, Marcus Vetter, Rosemarie Kell, Soile Tapio, Juliane Merl-Pham, Stephan M. Huber, Lena Edalat, Vanja Radulović, Nataša Anastasov, Michael J. Atkinson & Simone Moertl

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Radiation alters the cargo of exosomes released from squamous head and neck cancer cells to promote migration of recipient cells

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Radiation is a highly efficient therapy in squamous head and neck carcinoma (HNSCC) treatment. However, local recurrence and metastasis are common complications. Recent evidence shows that cancer-cell-derived exosomes modify tumour cell movement and metastasis. In this study, we link radiation-induced changes of exosomes to their ability to promote migration of recipient HNSCC cells. We demonstrate that exosomes isolated from irradiated donor cells boost the motility of the HNSCC cells BHY and FaDu. Molecular data identified enhanced AKT-signalling, manifested through increased phospho-mTOR, phospho-rpS6 and MMP2/9 protease activity, as underlying mechanism. AKT-inhibition blocked the pro-migratory action, suggesting AKT-signalling as key player in exosome-mediated migration. Proteomic analysis of exosomes isolated from irradiated and non-irradiated BHY donor cells identified 39 up- and 36 downregulated proteins. In line with the observed pro-migratory effect of exosomes isolated from irradiated cells protein function analysis assigned the deregulated exosomal proteins to cell motility and AKT-signalling. Together, our findings demonstrate that exosomes derived from irradiated HNSCC cells confer a migratory phenotype to recipient cancer cells. This is possibly due to radiation-regulated exosomal proteins that increase AKT-signalling. We conclude that exosomes may act as driver of HNSCC progression during radiotherapy and are therefore attractive targets to improve radiation therapy strategies.

Radiotherapy is a widely used treatment modality for head and neck cancer. However, radiation resistance, local recurrence as well as distant metastasis are commonly encountered treatment complications[1]. There are indications that the radiation treatment itself may increase the motility of glioblastoma, lung and head and neck cancer cells, thus influencing invasion capacity and the migration to local and distant sites[2–4]. In accordance, head and neck cancer patients had a significant higher incidence of distant metastasis if they received preoperative radiotherapy, although the overall survival was not affected[5]. Furthermore, *in vitro* studies found that irradiation increased cellular migration in head and neck cancer cell lines[6,7]. These findings suggest that radiation may promote the acquisition of a more motile phenotype in head and neck cancer cells. However, neither key components nor the underlying mechanisms of this phenomenon are fully understood.

Exosomes are a candidate to stimulate local tumour cell movement and pre-metastatic niche formation[8,9]. Exosomes are nanometer-sized, extracellular vesicles that are released from almost all cell types through the fusion of endosomal multivesicular bodies (MVBs) with the plasma membrane. They contain a variety of biomolecules including RNA, DNA, lipids and several different classes of proteins (e.g. signalling molecules, membrane trafficking proteins, cytoskeleton proteins, adhesion molecules, chaperones, enzymes)[10]. Protein loading

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is regulated by endosomal sorting complexes required for transport (ESCRT), tetraspanins and lipid-mediated processes, while RNA loading seems to depend on specific sequence motifs and interaction with RNA-binding proteins[11]. Cellular stress, including ionizing radiation, induces changes in the abundance of these exosomal molecules[12–14].

Released exosomes can interact with recipient cells either by ligand-receptor interaction and induction of intracellular signalling pathways after surface attachment or they can be incorporated by endocytosis or direct fusion resulting in the delivery of their cargo[15,16]. Subsequently, the exosomal cargo is functional within recipient cells and can modify their physiological state[17–20].

In a previous study we have demonstrated that exosomes modulate the radioresistance of head and neck cancer cells, indicated by higher survival and accelerated DNA repair in cells treated with exosomes isolated from irradiated cells[21]. Addressing the clinically relevant observation of radiation effects on local tumour recurrence and metastasis, we investigated if exosomes released from irradiated and non-irradiated cells differentially affect the migratory potential of HNSCC cells and if the radiation-induced changes in the exosomal cargo may trigger these effects (Fig. 1a).

Results

Exosomes from irradiated cells promote migration and increase chemotaxis-induced motility. Exosomes were isolated from the conditioned medium of irradiated or non-irradiated BHY squamous head and neck carcinoma cells by differential ultracentrifugation. Exosomes either purified from irradiated (EXO 6 Gy) or non-irradiated (EXO 0 Gy) cells showed the expected enrichment of the exosome marker proteins ALIX and TSG101 over cellular lysates. GAPDH was weakly detected in exosome lysates while it was highly abundant in cellular fractions. Calnexin, a protein not present within exosomes, was absent in exosome lysates, but showed a strong abundance in the cellular lysates (Fig. 1b). Furthermore transmission electron microscopy and nanoparticle tracking analysis confirmed homogeneous exosome preparations with a major population at an average size of 100–130 nm (Supplementary Fig. S1).

To study the influence of exosomes on cell migration we performed a gap-closure assay. BHY cells expressing green fluorescent protein (BHY-GFP) were preincubated with BHY exosomes isolated from either non-irradiated (EXO 0 Gy) or irradiated (EXO 3 Gy, EXO 6 Gy, EXO 9 Gy) cells. Figure 2a depicts a time course of the cellular movement of BHY-GFP cells. Cells preincubated with exosomes isolated from 6 Gy (EXO 6 Gy) and 9 Gy (EXO 9 Gy) irradiated cells closed the gap faster than cells incubated with exosomes from non-irradiated cells, indicating a migration stimulatory effect of exosomes from the irradiated cells. A lower radiation dose of 3 Gy (EXO 3 Gy) did not result in an enhanced migration, indicating that a pro-migratory response of exosomes is dose-dependent for head and neck cancer cells (Fig. 2a and b). To test if the observed exosome-stimulated migration is a ubiquitous phenomenon for head and neck cancer, we analysed the migratory behaviour of FaDu head and neck cancer cells after exosome incubation. Exosomes from irradiated FaDu cells boosted the migration of FaDu-GFP cells compared to exosomes from non-irradiated cells (Supplementary Fig. S2). Exosomal crosstalk between BHY and FaDu was studied by analysing the effect on the migration potential after exosome cross-transfer. Indeed, exosomes isolated from irradiated FaDu cells induced the migration of BHY-GFP cells and exosomes from irradiated BHY cells increased the motility of FaDu-GFP cells (Supplementary Fig. S2). Furthermore, we studied the radiation-induced migration effect of exosomes derived from non-tumour cells. Exosomes isolated from irradiated fibroblasts increased the motility of BHY-GFP cells, but to a lesser extent than exosomes from irradiated head and neck cancer cells. However, exosomes isolated from endothelial cells did not affect the migratory behaviour of BHY-GFP cells (Supplementary Fig. S2).

Additionally, we examined if exosomes are influencing motility by altering chemotaxis. The impedance, as a measure of transfilter migration, was more rapidly increased for BHY cells incubated with exosomes isolated from 6 Gy and 9 Gy irradiated BHY cells in comparison to cells treated with exosomes from non-irradiated cells (Fig. 3a). The slope of the migration curve confirmed that these exosomes augment the chemotactic phenotype (Fig. 3b). In contrast, exosomes from 3 Gy irradiated cells did not affect the chemotactic motility (Fig. 3a and b).

Exosomes from irradiated head and neck cancer cells trigger the AKT-pathway. One key regulator of migration processes in head and neck cancer is AKT-signalling[22,23]. To examine a potential effect of exosomes on AKT-pathway regulation the downstream target mTOR was analysed after 3 and 24 hours of exosome incubation. mTOR is predominantly phosphorylated at Ser2448 in response to stimuli which activate AKT[24] and is a mediator of pro-migratory signals in head and neck cancer[25–27]. The phosphorylation on Ser2448 of mTOR was increased at both time points after transfer of exosomes isolated from 6 Gy irradiated cells, compared to exosomes from non-irradiated cells (Fig. 4a, Supplementary Fig S3). This effect can be abrogated if endocytosis of exosomes is inhibited by Dynasore (Fig. 4b).

Furthermore, the phosphorylation level on Ser240/244 of S6 Ribosomal Protein (rpS6), a downstream target of the mTOR-signalling[28], was increased 24 hours after transfer of exosomes isolated from irradiated cells (Fig. 4c).

The increased motile phenotype of head and neck cancer cells receiving exosomes from irradiated cells was accompanied by increased matrix metalloproteinase (MMP) activity. MMP2 and MMP9 are both downstream targets of the AKT signalling and drive cellular motility[29–31]. Cells treated with exosomes from 6 Gy irradiated cells, released significantly more MMP2 and MMP9 in the supernatant, compared to cells supplemented with exosomes from non-irradiated cells (Fig. 4d).

The increase in mTOR-, rpS6-phosphorylation and MMP activity suggest that exosomes from irradiated cells are able to activate the AKT-signalling pathway in recipient cells.

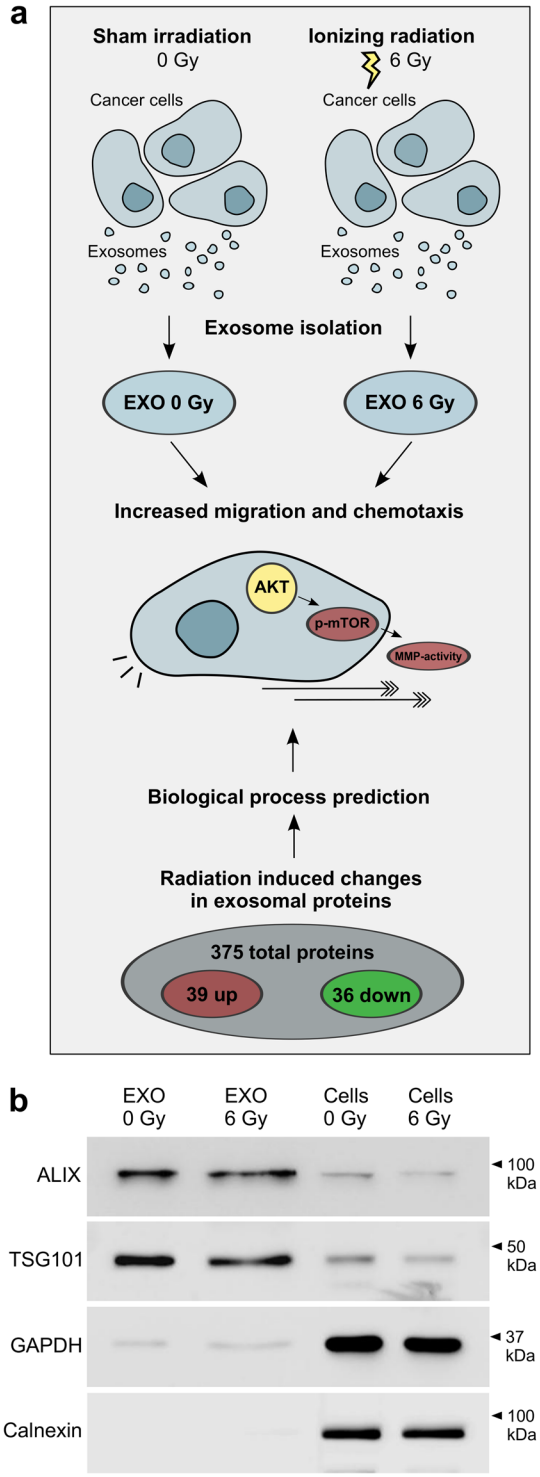

Figure 1. Functional and molecular comparison of exosomes released from 6 Gy irradiated and non-irradiated head and neck cancer cells. Exosomes isolated from irradiated BHY cells induce migration and chemotaxis by activating AKT-signalling and extracellular MMPs. In the same line radiation-induced changes of exosomal proteins predict effects on migration, chemotaxis and AKT-signalling. (**b**) Representative, cropped western blot of exosome markers ALIX and TSG101 as well as cytosolic markers GAPDH and Calnexin for BHY exosomes and cells isolated 24 hours after 0 and 6 Gy irradiation.

AKT-pathway is required for exosome-mediated migration after ionizing radiation. The AKT-inhibitor Afuresertib was used to block AKT activity. Indeed treatment with 5 μM Afuresertib caused reduced levels of phosphorylated mTOR, confirming that Ser2448 phosphorylation of mTOR is triggered by

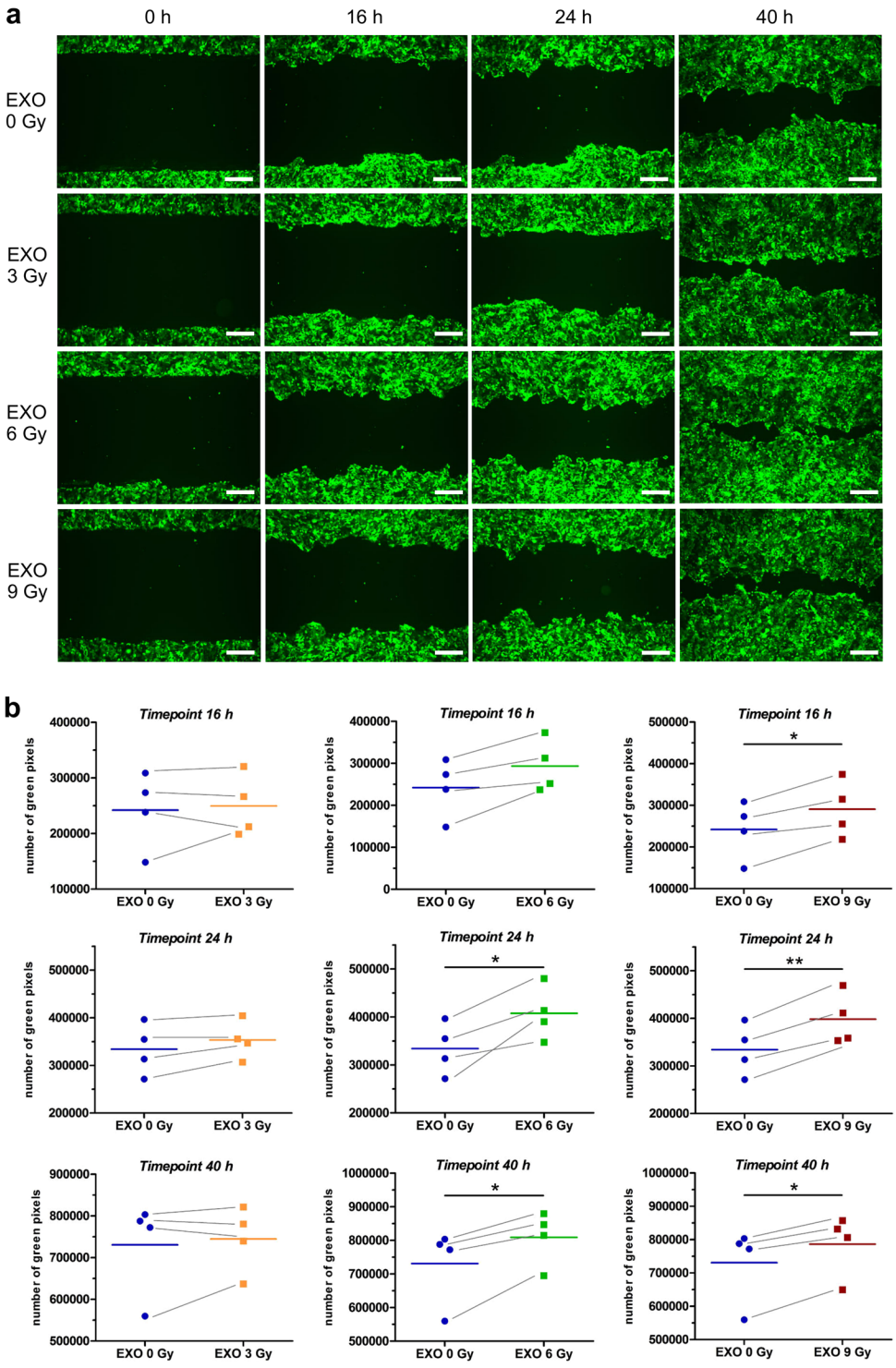

Figure 2. Exosomes from irradiated BHY cells enhance the migratory phenotype. (**a**) Exemplary wound healing of BHY-GFP cells after 16, 24 and 40 hours (scale bar: 500 µm). Cells were either preincubated with exosomes from non-irradiated (EXO 0 Gy), 3 Gy (EXO 3 Gy), 6 Gy (EXO 6 Gy) or 9 Gy (EXO 9 Gy) irradiated BHY cells. (**b**) Quantification of the wound healing capacity with the Image Colour Analyser after 16, 24 and 40 hours [n = 4; two-sided, paired t-test; p-value < 0.05].

AKT in BHY cells (Supplementary Fig. S3). Moreover AKT-inhibition reduced the migration of BHY-GFP cells in comparison to the control DMSO-treated cells (Supplementary Fig. S3). Combination of Afuresertib with exosome incubation was able to prevent the pro-migration effect of exosomes isolated from 6 Gy irradiated cells

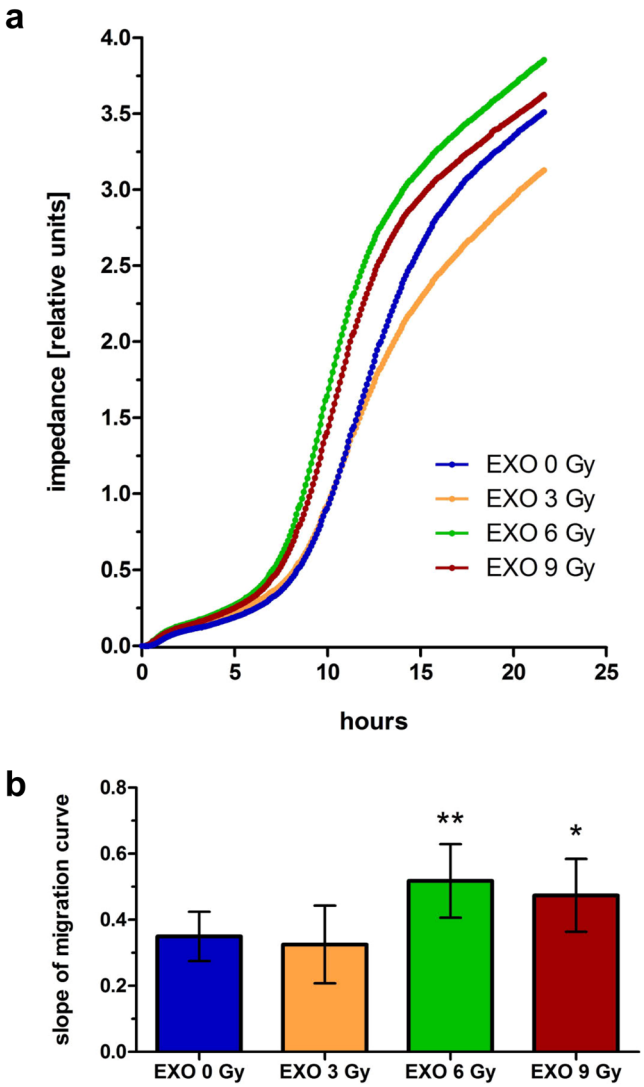

Figure 3. Exosomes from irradiated BHY cells enhance the chemotaxis-induced motility. The xCELLigence system was used to analyse the chemotactic movement of cells after a 24 hours pretreatment with exosomes from non-irradiated (EXO 0 Gy), 3 Gy (EXO 3 Gy), 6 Gy (EXO 6 Gy) or 9 Gy (EXO 9 Gy) irradiated BHY cells. (**a**) Mean impedance as measure of transfilter migration of cells is plotted over time. (**b**) Slope of the migration curves [n = 3; ± SD; two-sided, unpaired t-test; *p-value < 0.05; **p-value < 0.01].

(Fig. 5a and b). Inhibition of AKT with Afuresertib reduced the activity of MMP2 and MMP9, indicating that the MMP activity is AKT-dependent in BHY cells (Supplementary Fig. S3).

Exosomes from donor head and neck cancer cells transfer proteins to recipient cells. To further understand the role of exosomes in modifying migration capacity of recipient cells, we studied their ability to transfer exosomal proteins. BHY-derived exosomes were labelled with carboxyfluorescein succinimidyl diacetate ester (CFSE) and added to recipient BHY cells. Uptake and cytoplasmic distribution of the labelled proteins was visible 24 hours after exosome transfer to recipient cells, confirming that exosomes serve as an efficient tool for protein exchange between BHY cells (Fig. 6a, Supplementary Video S1). The control, PBS plus CFSE, did not display any fluorescence. The preincubation with the dynamin inhibitor Dynasore blocked the protein uptake, which suggests endocytosis as major exosome uptake mechanism in BHY cells (Supplementary Fig S4). We also studied exosome communication between different cell lines. Indeed FaDu cells took up BHY exosomal proteins and BHY cells absorbed exosomal proteins derived from FaDu cells. Furthermore, exosomal proteins from non-tumour fibroblasts and endothelial cells were transferred to both head and neck cancer cell lines (Supplementary Fig. S4). Exosomes are therefore potent vehicles to transfer proteins between same and different cell types.

Exosomal proteome of BHY cells. In order to examine whether exosome cargo contains proteins involved in induction of cell migration, we analysed exosomes that were isolated from BHY cells 24 hours after irradiation with 0 or 6 Gy with label-free quantitative proteomics. A total of 375 proteins were detected in the isolated

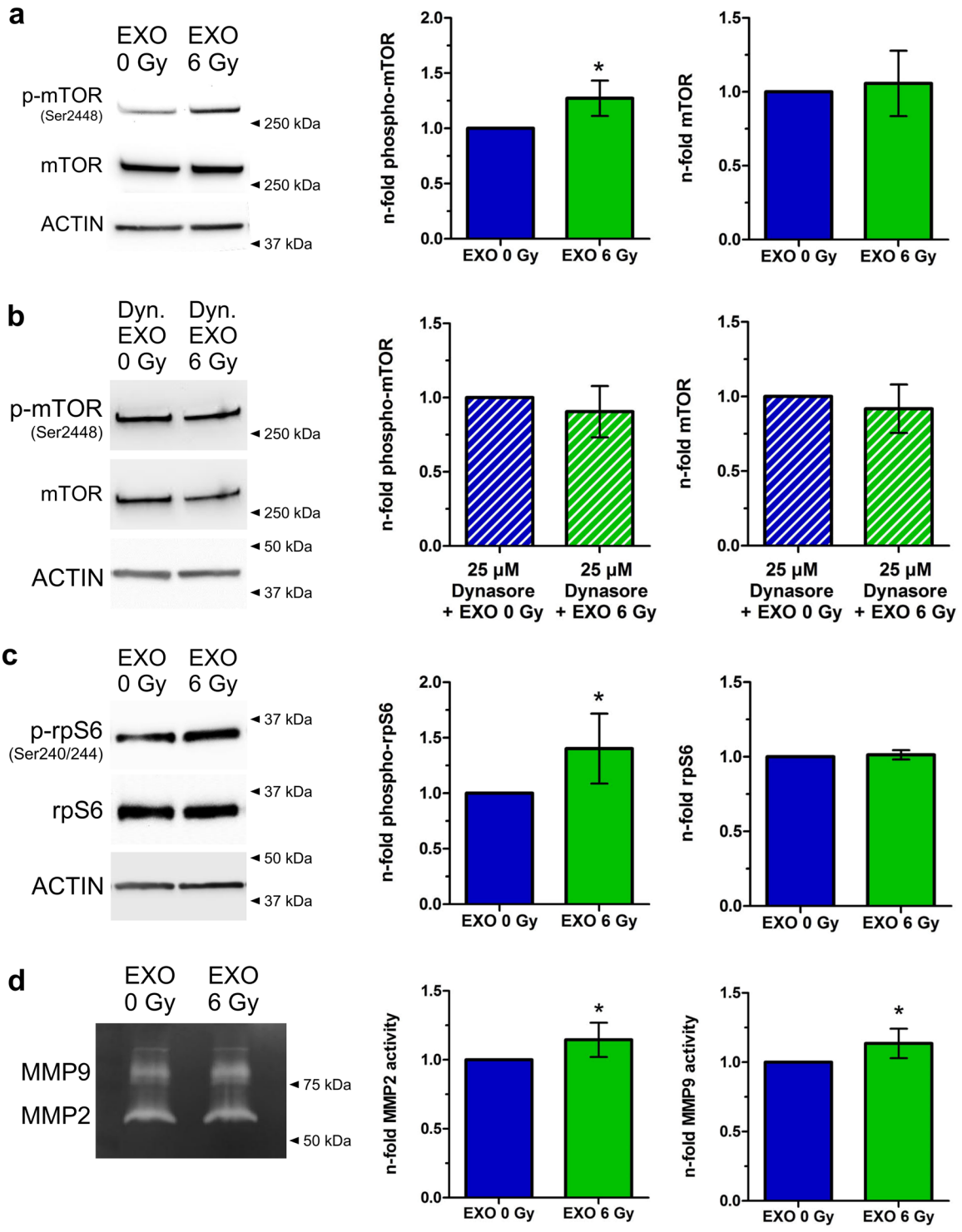

Figure 4. Exosomes from irradiated cells activate the AKT-pathway. (**a**) Western blot of phospho-mTOR (Ser2448) and mTOR of cells which were incubated for 24 hours with exosomes isolated either from irradiated cells (EXO 6 Gy) or from non-irradiated cells (EXO 0 Gy). Normalization was performed to ACTIN and to cells treated with exosomes from non-irradiated cells (EXO 0 Gy). Cropped blots are displayed [n = 4; ± SD; two-sided, one-sample t-test; p-value < 0.05]. (**b**) Western blot of phospho-mTOR (Ser2448) and mTOR of cells which were pretreated for 1 hour with 25 µM Dynasore and incubated for 24 hours with exosomes isolated either from irradiated cells (EXO 6 Gy) or from non-irradiated cells (EXO 0 Gy). Normalization was performed to ACTIN and to cells treated with exosomes from non-irradiated cells (EXO 0 Gy). Cropped blots are displayed [n = 3; ±SD; two-sided, one-sample t-test]. (**c**) Western blot of phospho-S6 Ribosomal Protein (Ser240/244) and S6 Ribosomal Protein of cells which were incubated for 24 hours with exosomes isolated either from irradiated cells (EXO 6 Gy) or from non-irradiated cells (EXO 0 Gy). Normalization was performed to ACTIN and to cells treated with exosomes from non-irradiated cells (EXO 0 Gy). Cropped blots are displayed [n = 7;

±SD; two-sided, one-sample t-test; p-value < 0.05]. (**d**) MMP2 and MMP9 activity in the supernatants 24 hours after transfer of exosomes isolated from irradiated (EXO 6 Gy) and from non-irradiated cells (EXO 0 Gy) on BHY cells. Normalization was performed to cells treated with EXO 0 Gy. Cropped gels are displayed [n = 6; ±SD; two-sided, one-sample t-test; p-value < 0.05].

exosomes. All proteins identified in BHY exosomes are listed in Supplementary Table S1. The detected proteins are grouped by STRING software *in silico* analysis into the compartments 'extracellular vesicle' and 'extracellular exosome' with a false discovery rate (FDR) of $1.1e^{-156}$–$3.5e^{-156}$ (Supplementary Table S2). A comparison between all identified exosomal proteins of BHY cells and the top 50 (most often detected) exosomal proteins listed in the ExoCarta global exosomal protein database showed an overlap of 86% (Supplementary Table S3). These findings support the conclusion about a conserved subset of exosomal proteins across cell types.

The composition of the exosomal protein cargo is modified following exposure to ionizing radiation. The comparison of exosomes isolated from non-irradiated donor cells (EXO 0 Gy) and exosomes isolated from irradiated donor cells (EXO 6 Gy) revealed that exposure to ionizing radiation modifies the protein content of exosomes secreted by the head and neck cancer cell line BHY. We found 39 proteins up- and 36 proteins downregulated in exosomes isolated from irradiated donor cells compared to exosomes isolated from non-irradiated cells (q-value < 0.05). All deregulated proteins are depicted in Fig. 6b and c and are listed in Supplementary Table S4. Remarkably, several proteins were highly upregulated with a maximal enrichment up to 57-fold.

***In silico* analysis of radiation-regulated exosomal proteins.** STRING analysis on protein function, as based on the number of network edges (196 compared to 77 for a random set of proteins; PPI (protein-protein-interaction) enrichment p-value $< 1 \times 10^{-15}$), revealed a high degree of protein interaction amongst the deregulated proteins (Supplementary Fig. S5). This suggests cooperative functions of the deregulated proteins. Indeed, the radiation-regulated exosomal proteins have a predicted influence on 142 biological processes (Supplementary Table S5). A considerable number of identified processes relate to cellular motility. Wound healing (FDR = $3.81e^{-9}$), locomotion (FDR = 0.0002), biological adhesion (FDR = 0.0004), regulation of cellular component movement (FDR = 0.0005), chemotaxis (FDR = 0.0005) and regulation of cell motility (FDR = 0.0006) were highly predicted to be influenced by the deregulated exosomal proteins released by irradiated cells. In addition Cytoscape pathway enrichment analysis of the deregulated proteins predicted an influence on PI3K-AKT-signalling (FDR = 0.0071) (Supplementary Table S6). Taken together, these results suggest that the radiation-deregulated exosomal proteins may play a role in inducing cellular motility via AKT activation.

Discussion

Radiation therapy may increase the invasive and metastatic properties of head and neck tumours[5–7]. In this study, we show that exosomes isolated from irradiated squamous head and neck cancer cells promote AKT-dependent migration and chemotaxis-induced motility in recipient cancer cells. Analysis of the exosomal cargo suggests that radiation-induced changes in the exosomal proteins increase migration via the AKT-pathway. As a consequence exosome-mediated cell-to-cell communication during radiotherapy may promote cancer cell motility.

To improve metastasis-free survival, it is essential to understand the underlying mechanism of radiation-induced cell migration. Our data demonstrate that exosomes from irradiated donor cells boost the motility in head and neck cancer cells. Interestingly this effect depends on the irradiation dose applied to the exosome donor cells and suggests dose-dependent alterations in the exosome-mediated cell-to-cell communication. Importantly, migration effects cannot be assigned to differences in proliferation capacity, since we showed equal effects on proliferation of recipient cells which were treated with exosomes isolated from irradiated compared to non-irradiated donor cells[21]. In accordance Arscott *et al.* showed augmented migration of glioblastoma cells after pretreatment or chemotactic stimulation with exosomes isolated from 4 Gy irradiated cells[32].

Additional evidence for the motility promoting effect of exosomes from irradiated cells comes from our observations that exosomes isolated from irradiated donor cells trigger the AKT-pathway in the recipient cells (evidenced by increased p-mTOR and p-rpS6). The AKT-pathway is the most frequently mutated oncogenic pathway in head and neck cancer, a key regulator of radiation resistance and a major driver of cellular movement and migration processes[22,33–35]. The impact of AKT-signalling on the migration process was confirmed by AKT-inhibition with Afuresertib. Inhibitor treated cells exhibit a reduced, but still existing migratory potential. The observation that exosomes from irradiated donor cells were incapable to compensate the effect of Afuresertib supports the key-role of the AKT-pathway as a regulator of exosome-stimulated migration after ionizing radiation. In line with this, a study from Pickhard *et al.* showed that inhibition of PI3K and mTOR activity with LY294002, respectively rapamycin, blocks the radiation-induced migration of BHY head and neck cancer cells[7]. Moreover, preclinical models and clinical trials already demonstrated that AKT- and mTOR-inhibitors are promising antitumour agents, which might increase the efficacy of radiotherapy and therefore patient survival[22,36].

AKT induces migration processes through the regulation of MMP activity, which is critical for the degradation of the extracellular matrix[37,38]. Dysregulation of MMP2 and MMP9 is frequently present in head and neck cancers and is associated with lymph node metastasis and poor prognosis[39,40]. Moreover, Park *et al.* identified ionizing radiation as the trigger for increased AKT-pathway induction combined with enhanced MMP2 activity in glioma cells[29]. We have found more active MMP2 and MMP9 to be released after incubation with exosomes from irradiated cells. This supports our suggestion that enhanced AKT-signalling promotes the increased migration

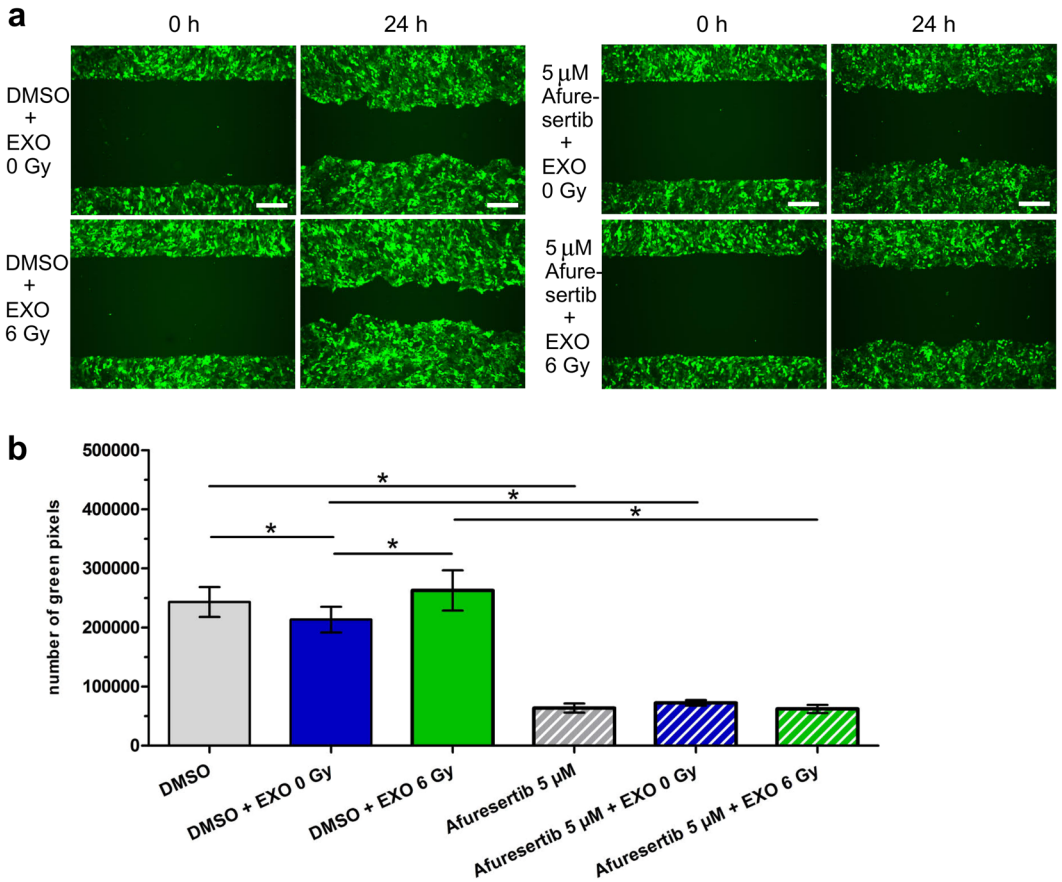

Figure 5. Exosomes from irradiated cells activate the AKT-pathway to induce migration. (**a**) Exemplary wound healing of BHY-GFP cells after treatment with 5 µM of the AKT-inhibitor Afuresertib or DMSO, in combination with exosomes isolated from irradiated (EXO 6 Gy) and from non-irradiated cells (EXO 0 Gy). The pictures were taken 24 hours after migration start (scale bar: 500 µm). (**b**) Quantification of wound healing capacity with the Image Colour Analyser 24 hours [n = 3; ±SD; two-sided, paired t-test; p-value < 0.05].

through degradation of the extracellular matrix by fine-tuning MMP activity. In a clinical context MMP2 and MMP9 overexpression may be helpful markers in diagnosing head and neck cancer metastasis[41].

Previous studies showed that external stimuli and stress conditions, including ionizing radiation, are reflected by changes in the exosome composition[14,32,42]. Our proteomic analysis also revealed that radiation induces changes in the exosomal protein content. According to bioinformatics analysis these protein changes have the potential to influence migration processes as well as AKT-signalling in recipient cells. Based on our CFSE labelling results and on previous findings that demonstrated the transfer of proteins by exosomes and their influence on cell fate in the recipient cells[17,18,20], we suggest that exosomes from irradiated cells might transfer proteins to recipient cells that increase cellular motility by AKT activation and MMP release. The abrogation of increased p-mTOR levels after incubation with EXO 6 Gy and simultaneous blockage of exosome internalization by dynamin-inhibition suggests that rather the transfer of cargo than exosome surface interactions induce the observed effects.

Candidate proteins which were upregulated in exosomes after irradiation, that activate AKT, stabilize MMP2[43], enhance exosome-mediated motility[44] as well as metastasis[45,46] and invasion[47] are FGFR1, HSP90AA1, HSP90AB1, HSP90B1 and VTN. The second most upregulated protein FGFR1 (53-fold) is overexpressed in 75% of HPV-negative patients with HNSCC, correlates with poor overall and disease-free survival[48], increases the metastatic potential[45] and induces radiation resistance[49]. Nonetheless, a cooperative function of several exosomal proteins is highly conceivable.

In summary, exosomes derived from irradiated head and neck cancer cells are able to confer a migratory phenotype to recipient cancer cells via increased AKT-signalling. Our proteomic data suggest a subset of radiation-regulated exosomal proteins as candidates to induce the pro-migratory effects, however we cannot exclude effects of other exosomal components. In a clinical view exosome-mediated cell-to-cell communication may act as potential driver of metastatic head and neck cancer progression during tumour radiation treatment and therefore represents an attractive target to improve radiation therapy strategies.

Materials and Methods

Cell lines and irradiation. The human cell lines BHY (DSMZ no.: ACC 404) and FaDu (ATCC®HTB43™) are squamous cell carcinoma of the head and neck region. BHY cells were cultivated in DMEM (Dulbecco's modified Eagle's medium, Gibco) with high Glucose, 2 mM L-Glutamine and sodium pyruvate at 10% CO_2, whereas

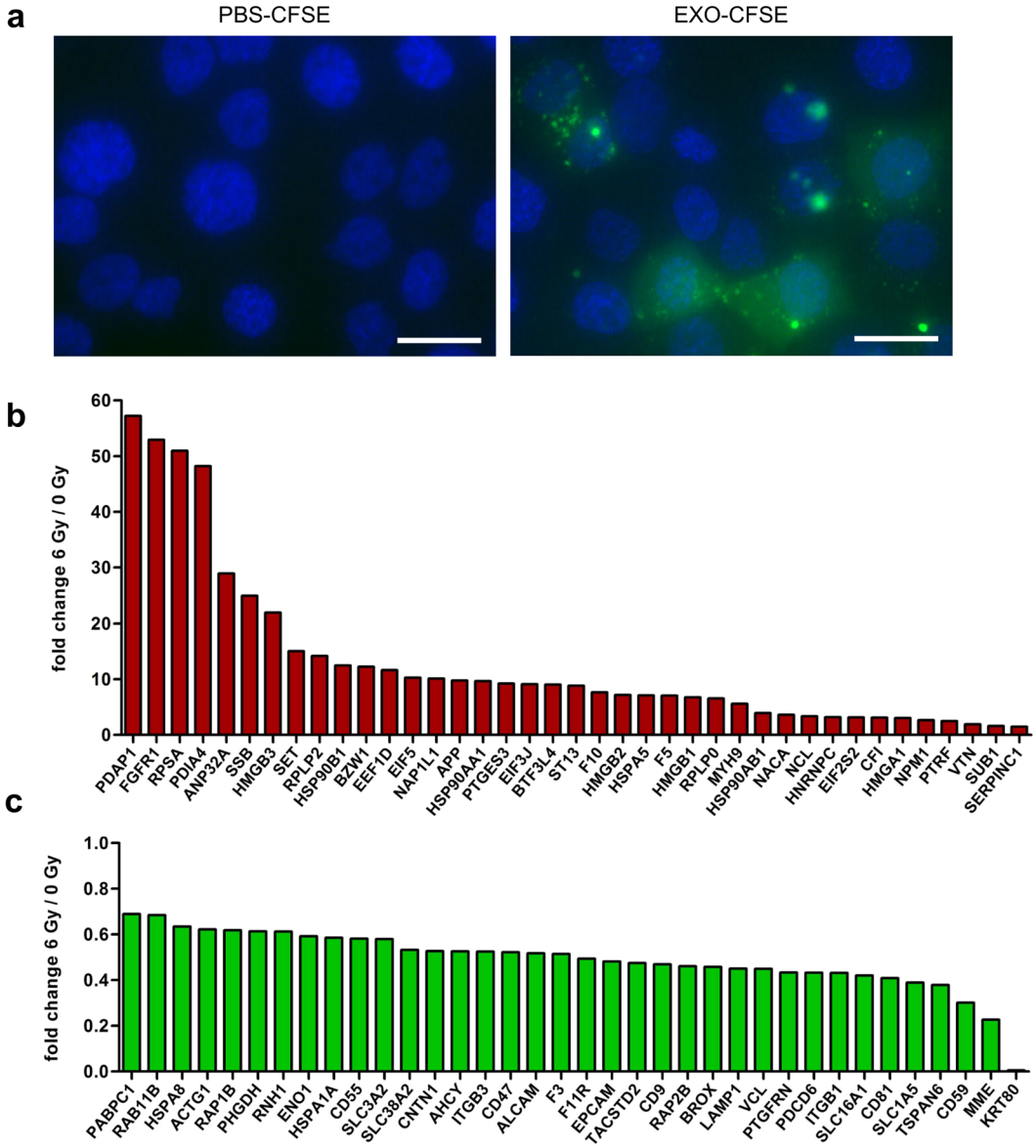

Figure 6. Exosomes from head and neck cancer cells transfer proteins to recipient cells and have a modified protein composition after ionizing radiation. (**a**) Exosomal proteins (EXO-CFSE) of BHY cells and PBS (PBS-CFSE) as negative control were stained with CFSE and subsequently transferred onto recipient BHY cells. The protein uptake was monitored after 24 hours of exposure (scale bar: 25 µm). Protein analysis of exosomes isolated 24 hours after 6 Gy irradiation of the head and neck cancer cell line BHY revealed (**b**) 39 upregulated and (**c**) 36 downregulated proteins [n = 3; FDR-adjusted p-value (q-value), ≥2 unique peptides, fold-change between ≤0.7 and ≥1.3; q-value < 0.05.

FaDu cells were cultivated in DMEM (GE Healthcare) with low glucose, 2 mM L-Glutamine and 25 mM HEPES at 5% CO_2. For both cell lines medium was supplemented with 10% FCS (foetal calf serum, Bio&SELL). The human skin fibroblast cells 1BR3 (ECACC 90011801) were maintained in DMEM with low glucose and 15% FCS at 5% CO_2. The human coronary artery endothelial cells HCAEC (ATCC® PCS-100–020™) were cultivated in MesoEndo Cell Growth Medium Kit (Cell Applications) at 5% CO_2. All cells were incubated in a humidified atmosphere at 37 °C.

BHY-GFP and FaDu-GFP cells (expressing green fluorescence protein) were established by lentiviral transduction using pGreenPuro transfer vector (SBI, CA, USA) and previously described lentiviral protocols[50,51]. For stable and constitutive GFP expression cells were cultivated in DMEM medium containing 0.3 µg/ml or 0.1 µg/ml puromycine for BHY-GFP or FaDu-GFP cells, respectively.

For AKT-inhibition BHY or BHY-GFP cells were treated for 3 or 24 hours with 5 µM of Afuresertib (GSK2110183; Cell Signaling Technology). The Dynamin inhibitor Dynasore (25 µM, CAS 304448-55-3, Sigma) was added to BHY cells 1 hour before exosome treatment. Control cells were sham-treated with DMSO (Sigma-Aldrich).

Cell line identification was confirmed by Eurofins Genomics (sequencing of nine different loci: D5S818, D13S317, D7S820, D16S539, VWA, TH01, AM, TPOX, CSF1PO). Mycoplasma negative status was confirmed with MycoAlert.

A ^{137}caesium source (HWM-D2000, Wälischmiller Engineering) was used to irradiate the cells with γ-rays at a dose rate of 0.45 Gy per min.

Isolation of exosomes. Exosomes were isolated from culture supernatants by a serial centrifugation procedure as previously described[21]. Briefly, cells were seeded in 10 cm dishes and fresh medium with exosome-depleted FCS (edFCS) was added prior to irradiation. After 24 hours of cultivation, the medium was collected, centrifuged at 10,000 g for 30 minutes at 4 °C and passed through a filter with a pore size of 0.22 µm. The filtrate was centrifuged with 100,000 g for 75 minutes at 4 °C. The supernatant was discarded and the exosome pellet was resuspended in PBS. Another round of ultracentrifugation (100,000 g, 75 minutes, 4 °C) was applied and the final exosome pellet resuspended in fresh PBS. To determine the biological activity of exosomes we incubated recipient cells with exosomes in medium supplemented with edFCS and exosome preparations isolated from irradiated and non-irradiated donor cells. Applied exosome amounts correspond to a three-fold concentration of exosomes compared to native conditions. Exosomes were stored at −20 °C. Cells were harvested using a cell scraper and stored at −20 °C.

For the preparation of edFCS, bovine exosomes were removed from foetal calf serum by centrifugation at 100,000 g and 4 °C for 14 hours.

Electron microscopy. BHY and FaDu exosomes (isolated from 3 ml conditioned medium) were absorbed onto glow discharged carbon coated grids (G2400C from Plano) for 2 minutes. The solution was blotted of and negatively stained with 4% ammonium molybdate (Sigma-Aldrich) solution for 30 seconds. Micrographs were recorded with a Jeol JEM 100CX electron microscope at 100 kV onto Kodak SO163 film. Negatives were digitized with a Hasselblad Flextight × 5 scanner at 3000 dpi, resulting in a pixel size of 0.25 nm/px. For visualization images were binned to 1 nm/px.

Exosome size. Exosome size distribution was analysed by using the NanoSight LM10 (Malvern) microscope. Exosome preparations (isolated from 2.5 ml conditioned medium) were diluted 1:600 with H_2O to achieve 15 to 50 particles per frame for tracking. Each sample was analysed three times for 30 seconds.

Migration assay. Gap-closure (wound healing) was performed with GFP labelled cells. Silicon grids (Ibidi) with 12 rectangular wells and a wall size of 2 mm were placed air bubble-free in 10 cm cell culture dishes. 42,000 BHY-GFP or 60,000 FaDu-GFP cells were then seeded per well. After cell attachment the medium was discarded and replaced by exosome-depleted medium. Subsequently cells were pretreated with exosomes, 5 µM of the AKT-inhibitor Afuresertib (GSK2110183; Cell Signaling Technology) or DMSO (Sigma-Aldrich). After 24 hours the medium was discarded and the silicon grids were removed carefully to generate a defined gap (2 mm) in the monolayer. 8 ml of exosome-depleted medium, medium containing 5 µM Afuresertib or DMSO were added. Starting pictures (0 hour) were taken immediately after grid removal and repeated after 16, 24, 40 and 48 hours to monitor migration. For quantification Adobe Photoshop CS5 (Adobe Systems) was used to identify green fluorescent cells from the starting picture (0 hour) and to subtract this area in pictures from later time points. Finally the program Image Colour Analyser (developed by Marcus Vetter; source code available upon request) was used to quantify the migratory potential. This tool analyses the green colour value in an intensity range from 0 to 255 (RGB-range) for each pixel of the picture and allows the calculation of the total pixel number that exceed a given green value.

Chemotaxis-induced motility. The xCELLigence® Real-Time Cell Analyser (RTCA) DP System (Roche) was used to measure gradient-driven cell movement. BHY cells were pretreated for 24 hours with exosomes in medium containing edFCS. Then cells were re-plated into CIM-plates (Roche Diagnostics) with 8 µm pores. In total 60,000 cells in 1% edFCS-containing medium were seeded into the upper chamber, while the lower chamber contained 10% edFCS as chemoattractant. Chemotaxis-induced migration was tracked in real-time over a time span of 24 hours in the RTCA DP instrument at 37 °C with 10% CO_2. The increase in impedance measured on electrodes on the lower surface of the filter membrane reflects cell migration[4].

Zymography. To measure gelatinase activity, cell culture supernatants were collected 24 hours after exosome or Afuresertib (GSK2110183; Cell Signaling Technology) treatment and concentrated (5-fold) with centrifugal filter units (Amicon Ultra; 0.5; 100k). The BCA-assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) was applied according to the manufacturer's instructions to determine the protein concentration. Equal amounts of protein were treated with 5x non-denaturing sample buffer (4% SDS, 20% glycerol, 0.01% bromophenol blue, 125 mM Tris-HCl) and separated in a 10% polyacrylamide SDS gel containing 1 mg/ml gelatine (Sigma). After electrophoresis, the gel was washed twice with washing buffer (2.5% Triton X-100, 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) for 30 minutes, rinsed once in incubation buffer (1% Triton X-100, 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) and stored for 24 hours at 37 °C in the incubation buffer. A 5% Coomassie solution was added for 60 min to stain the gel. Destaining solution containing 40% methanol and 10% acetic acid was applied until gelatine digestion was visible as clear bands against the background. The detection camera FluorChem HD2 (Alpha Innotec) and the Alpha View Software (ProteinSimple) were used to image the gelatine digestion.

Quantification of exosomal and cellular proteins. Exosomes and cells were disrupted in lysis buffer II (25 mM Tris pH 7.5, 120 mM NaCl, 1% Triton X-100, 1% PSMF, 1 mM NOV, 1 mM Leupeptin) on ice. Exosomes

were lysed for 4 hours, while cells were incubated with the lysis buffer II for 1 hour. The protein concentration was determined by applying the BCA-assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) according to the manufacturer's instructions.

For immunoblotting 10 µg cellular protein and 10 µl exosome lysate (isolated from 3.5×10^6 cells) were used to run a standard western blot protocol. Antibodies directed against ALIX (2171, Cell Signaling), TSG101 (GTX70255, GeneTex), GAPDH (sc-47724, SantaCruz), Calnexin (sc11397, SantaCruz), p-mTOR Ser2448 (5536, Cell Signaling Technology), mTOR (2983, Cell Signaling), p-AKT Ser473 (9271, Cell Signaling Technology), p-S6 Ribosomal Protein Ser240/244 (2215, Cell Signaling), S6 Ribosomal Protein (2212, Cell Signaling) and ACTIN (SAB1305567, SIGMA-Aldrich Chemie) were applied. Secondary horseradish peroxidase-conjugated antibodies (1:40.000; anti-rabbit: sc2004 and anti-mouse: sc2005) and the chemoluminescence Amersham ECL reaction kit (GE Healthcare) were used for detection.

Trafficking of exosomes monitored with fluorescent labelled proteins. To monitor the exosome-mediated trafficking of proteins the Exo-Glow™ kit (System Biosciences), based on carboxyfluorescein succinimidyl diacetate ester (CFSE) chemistry, was applied with slight variation to the manufacturer's protocol. Exosomes were incubated with 1x Exo-Green for 10 minutes at 37 °C. To remove residual dye the samples were loaded on exosome-spin columns (Invitrogen) and processed according to the manufacturer's protocol. Exosomes with green fluorescent labelled proteins were transferred onto BHY cells. Nuclei staining was performed 24 hours later by adding NucBlue™ Live Cell Stain (Life Technologies). The uptake of the exosome-mediated proteins was monitored by fluorescence microscopy.

Quantitative proteomic analysis. Exosomal proteins were isolated by adding 20 µl of lysis buffer II (25 mM Tris pH 7.5, 120 mM NaCl, 1% Triton X-100, 1% PSMF, 1 mM NOV, 1 mM Leupeptin) to 40 µl of exosome suspension isolated from 1.5×10^7 cells. The samples were incubated for 4 hours on ice with repeated vortexing and the protein concentration was determined by the BCA assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) following the manufacturer's instructions.

Sample preparation, LC-MS/MS measurement, label-free quantitative analysis and database searches were performed as previously described[13]. Briefly, 5 µg of protein were digested using a modified filter-aided sample preparation (FASP), followed by the LC-MS/MS analysis performed on a LTQ OrbitrapXL (Thermo Fisher Scientific) coupled to an Ultimate3000 nano high-performance liquid chromatography system (Dionex). Alignment of peptides was set to at least 89.5% and single charged features as well as features with charges higher than +7 were eliminated. The Mascot search engine (Matrix Science, version 2.5.0) with the Ensembl Human database (version 83, 31236148 residues, 83462 sequences) was used for identification.

To identify significantly changed proteins a FDR-adjusted p-value (q-value) of three independent biological replicates was calculated. Here peptides with $\geq$2 unique peptides, a fold-change between $\leq$0.7 and $\geq$1.3 plus a q-value of $<$0.05 were considered as statistically significant deregulated.

In silico analysis was performed with several bioinformatics tools. The top exosomal protein candidates of ExoCarta, the web-based database of exosomal proteins, RNA and lipids, was used to compare the detected exosomal proteins from BHY cells with proteins recorded within exosomes ((http://exocarta.org/exosome_markers_new) accessed 09.03.2017)[52]. Protein subcellular localizations and functions were determined using STRING: *functional protein association networks* (http://STRING-db.org/)[53]. A pathway enrichment analysis (FDR $<$ 0.05) of the deregulated exosomal proteins was performed using the Reactome 5.1.0 application[54] in the Cytoscape 3.2.1 software[55].

Statistical analysis. Data show the mean of independent biological experiments with the standard deviation ($\pm$SD). The two-sided paired, unpaired or the one-sample t-test were used for statistical analysis and a p-value $<$ 0.05 was deemed statistically significant, while a p-value $<$ 0.01 was considered highly significant.

Data availability. The datasets generated during the current study are available from the corresponding author on reasonable request. The MSF files of the obtained MS/MS spectra can be found under STUDY1095 in https://www.storedb.org.

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Author Contributions

L.M., O.A., T.H., S.H., M.A., S.M. conceived and designed the experiments. L.M., K.W., R.K., J.M., L.E., V.R., S.M. performed the experiments. L.M., O.A., J.M., S.H., S.M. performed statistical analysis and figure processing. L.M., O.A., T.H., M.V. performed bioinformatics analysis. M.V., S.T., S.H., L.E., V.R., N.A. contributed reagents, materials and analysis tools. L.M., T.H., M.A., S.M., wrote the main manuscript text. All authors revised the manuscript.

Additional Information

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3 Conclusions and outlook

Exosomal communication influences cell fate after exposure to stress situations[76,78,79]. The here presented thesis demonstrates that ionizing radiation is capable of modifying the exosomal messaging between head and neck cancer cells. Thus, radiation is affecting exosomal release and uptake rates as well as the cargo composition. Furthermore, exosomes secreted from irradiated cells promote survival, DNA repair efficiency, wound healing and chemotaxis-induced motility of acceptor cells. The increase in cellular migration is influenced by activation of the AKT-signaling. Bioinformatics analyses of the exosomal cargo suggest that radiation-induced changes in the exosomal proteome serve to increase motility via AKT-pathway activation in recipient cells. Consequently, the radiation-induced and exosome-mediated response causes a radioresistant and motile phenotype in recipient HNSCC cells. In a clinical context, cell communication via exosomes might promote tumor survival and increase the invasive and metastatic properties in- and outside of the radiation field of head and neck cancers during radiotherapy. As a consequence, a detailed understanding of the underlying mechanisms of exosomes in the radiation response of HNSCC is essential to further optimize treatment strategies.

3.1 Influence of radiation on exosome release and uptake

Exosomes were isolated from irradiated and non-irradiated head and neck cancer cells via differential ultracentrifugation (1.5.1) and were characterized by transmission electron microscopy (TEM), immunoblotting, proteomic analysis, as well as nanoparticle tracking analysis (NTA) (1.5.2 and 1.5.4). The TEM displayed the expected nanometer-sized vesicles with a cup-shaped structure. To further address the quality of the exosomal preparations, the presence of exosomal marker proteins plus the absence of Calnexin were demonstrated in immunoblots. Moreover, a comparison between the exosomal proteins of BHY cells identified by mass spectrometry and the 50 most often detected exosomal proteins listed in the ExoCarta global exosomal protein database showed a broad overlap. The NTA confirmed the presence of exosomes in the preparations, and demonstrated the significantly increased abundance of head and neck cancer exosomes after 3 and 6 Gy irradiation. Ionizing radiation has been shown to increase the exosome release

from peripheral blood mononuclear cells (PBMCs), prostate cancer and glioblastoma cells[103,208,209].The release of microvesicles, a class of larger extracellular vesicles, is likewise enhanced in response to non-apoptotic doses of irradiation and hypoxia[210]. Not only the release but also uptake mechanisms are influenced by ionizing radiation. The here presented study provides evidence that head and neck cancer cells receiving an irradiation dose of 4 Gy take up more exosomes than non-irradiated cells. In non-cancerous cells Hazawa et al. showed that radiation increases the cellular uptake of exosomes through co-localization of the integrin CD29 and the tetraspanin CD81[211]. Beyond that, the study of Arscott et al. demonstrated increased uptake of exosomes which were isolated from irradiated glioblastoma cells[103]. The augmented release and uptake efficiencies after ionizing radiation suggest that the exosomal communication is highly relevant for the radiation stress response.

3.2 Influence of radiation on exosome composition

Besides modulating the amount of exosomes, radiation is affecting the vesicle composition. Elevated or reduced levels of miRNAs or long non-coding RNAs were previously detected in exosomes from irradiated cells[17,212,213]. For example, *in vitro* γ-irradiation of whole blood with 2 Gy changed the miRNA content of exosome-like vesicles, but affected also their protein composition[198]. The effect of radiation on the exosome protein profile of head and neck cancer cells was already studied by Jelonek et al. after 2 Gy exposure of FaDu cells. Ionizing radiation results in an increased level of exosomal proteins involved in transcription, translation, cell signaling and cell division. The radiation-induced exosomal cargo changes might mediate a dynamic adaption of cells exposed to stress conditions[214]. Changes in exosomal proteins involved in the radiation response relevant pathways were also discovered in this doctoral thesis by mass spectrometric analysis of BHY exosomes. From 375 identified exosomal proteins, 39 proteins were up- and 36 downregulated after irradiating BHY cells with 6 Gy. Bioinformatics analyses assigned these radiation-induced changes of the exosomal protein content to migration processes, stress responses as well as AKT-signalling. In line with the prediction tools, exosomes from irradiated cells promoted the migration by AKT-pathway activation, increased the cell survival and DNA repair capacity (1.3.3). This accordance in

composition changes and observed function of exosomes after ionizing radiation suggests specific radiation-induced cargo sorting mechanisms. However, the cellular processes and molecular mechanisms that induce sorting changes after radiation are not understood up to now.

3.3 Exosome-mediated biological effects in the context of ionizing radiation

3.3.1 Effect on proliferation and cell survival after irradiation

External stimuli like ionizing radiation change the intercellular signaling and cause phenotype adaptions of the exosomal acceptor cells (1.1.4). This thesis reports that exosomes from both non-irradiated and irradiated donor cells increase the proliferation of non-irradiated recipient cells and the survival of irradiated recipient cells. Hereby, exosomes from irradiated cells beneficially affect the cellular survival after ionizing radiation compared to exosomes from non-irradiated donor cells. A recent study from Cerreto et al. confirms that exosomes from irradiated neuroblastoma cells stimulated proliferation, and conferred radiation resistance to recipient cells irradiated with 0, 1 and 5 Gy[215]. Likewise, microvesicles from chronically irradiated primary human gliomas increased the cellular survival in recipient glioma cells[216]. Also exosomes and microvesicles from non-irradiated mesenchymal stem, bone marrow-derived or stromal cells, as well as gliomas, increased proliferation, survival and radiation resistance in recipient cells[211,216-218]. However, there are some contradictory reports showing that exosomes from irradiated non-cancerous cells reduced viability in keratinocyte cells[219] and induced autophagy in bronchial epithelial cells[212]. Nevertheless, the here presented study demonstrates in good agreement with previous findings that exosomes from irradiated cancer cells induce a radioresistant phenotype in recipient cells.

3.3.2 Effect on DNA repair mechanisms after irradiation

The role of the exosomal communication in the context of DNA repair mechanisms and genomic integrity after ionizing radiation was previously studied. Exosomes isolated from irradiated breast cancer cells increased the level of DNA damage in non-irradiated cells. This effect persisted for more than 20 doublings in the progeny of bystander cells, and demonstrated the long-lived effect of exosome signaling[203,220]. Fibroblast exosomes isolated after 2 Gy irradiation increased the

level of micronuclei and DSBs (53BP1 foci) in non-irradiated recipient cells[221]. Increased level of γH2AX and chromosomal aberrations were also found in murine splenocytes treated with bone marrow-derived extracellular vesicles derived from whole-body irradiated mice (0.1, 0.25 and 2 Gy)[222]. In contrast, extracellular vesicles from murine mesenchymal stem cells reduced the level of phosphorylated γH2AX and cleaved PARP after exposure to 5 Gy radiation. The authors suggest that the extracellular vesicles accelerated the DNA repair efficiency or reduced the initial DNA damage[218]. In the here conducted head and neck cancer study the exosome effect on DNA DSB repair mechanisms was investigated after irradiating both donor and recipient cells. While exosomes derived from non-irradiated cells retard the DSB repair process, exosomes from irradiated cells accelerate the DNA repair efficiency in irradiated recipients. The treatment with RNase and a detergent-peptidase-mixture abrogates the observed effects, suggesting that exosomal RNA molecules may promote the repair process in recipient cells. Moreover, the cross-transfer of exosomes between different cell lines confirms the origin-independent functionality of exosomes in cell-to-cell communication.

3.3.3 Effect of tumor cell migration after irradiation

Exosome signaling plays a pivotal role in cellular movement processes and metastatic spread[69]. Beer et al. demonstrated crosstalk between non-tumorigenic cells where exosomes from irradiated and non-irradiated PBMC equally enhanced fibroblast migration[208]. Conversely, microvesicles isolated from irradiated glioma and lung cancer cells increased MMP-2 activity or MMP-9 expression, thus, inducing the invasive and metastatic potential of recipient cells[210,216]. A detailed study from Arscott et al. demonstrated that exosomes from 4 Gy irradiated glioblastoma cells increased the migration potential in comparison to exosomes from non-irradiated cells. The exosomes, which function either as a chemoattractant or as a migration-activator after pre-incubation, exhibited elevated levels of migration-associated mRNAs (e.g. CTGF) and proteins (e.g. IGFBP2)[103]. In line with the study from Arscott et al. this doctoral thesis demonstrates that head and neck cancer exosomes isolated from irradiated cells promote the wound healing capacity and chemotaxis-induced migration. The origin-independent, unspecific functionality of exosomes was further demonstrated by cross-transfer of exosomes between different cell types. Not

only tumor-to-tumor signaling but also exosomes derived from irradiated fibroblasts increased the migration of head and neck cancer cells. Moreover, the altered exosomal proteome after head and neck cancer irradiation is enriched for migration processes and the induction of the pro-migratory AKT-signaling pathway. Indeed, the AKT downstream targets mTOR and rpS6 exhibited a higher phosphorylation level upon transfer of exosomes from irradiated cells. Exosomal uptake, rather than surface attachment, is responsible for the AKT-pathway induction, since inhibition of endocytosis with simultaneous exosome transfer did not result in the AKT-pathway activation. This result supports the assumption that the modulated exosomal protein cargo is in part responsible for the observed effects. Exosome transfer plus simultaneous AKT-inhibition with 5 µM Afuresertib blocked the exosome-mediated and radiation-induced migration potential. Thus, the AKT-pathway-activation is essential for the increased cellular motility evoked by exosome communication after irradiation. Previous work already demonstrated that the AKT-pathway can be activated by exosome signaling[170,223], and that it is a critical mediator for the radiation-induced and dose-dependent increase in head and neck cancer cell migration[116]. Furthermore, radiation and AKT induce migration processes through the regulation of MMP activity and degradation of the extracellular matrix[117,157,224]. Indeed, the activity of the released MMP-2 and MMP-9 was blocked by AKT-inhibition and increased after transfer of exosomes obtained from irradiated cells.

In summary, exosomes from irradiated head and neck cancer cells induce a motile phenotype by activating the AKT-pathway and modulating MMP-activity.

3.4 Summary and outlook

In conclusion, this thesis demonstrates that exosomal communication is a component of the radiation response of head and neck cancer cells. Moreover, exosomal signaling between the cells is intensified after ionizing radiation, and gains importance by mediating tumor cell protection and promoting cellular migration. Thus, exosomes are essential vehicles to deliver signals from irradiated cells to local and distant irradiated as well as to non-irradiated cells. Exosomes and their cargo are biologically active and cause the activation of intracellular signaling cascades. The cellular reprogramming allows the adaption of the cellular phenotype to new conditions and may lead to modifications of the extracellular environment. These

new insights highlight that head and neck cancer exosomes deliver an extensive message after irradiation. The intensified exosomal communication, the modified exosomal protein composition, the induction of pro-tumorigenic intracellular signaling cascades, the increased survival, DNA repair, migration and chemotaxis as well as MMP-activity confer radioresistance and tumor spread to the exosome-targeted head and neck cancer cells (Figure 5).

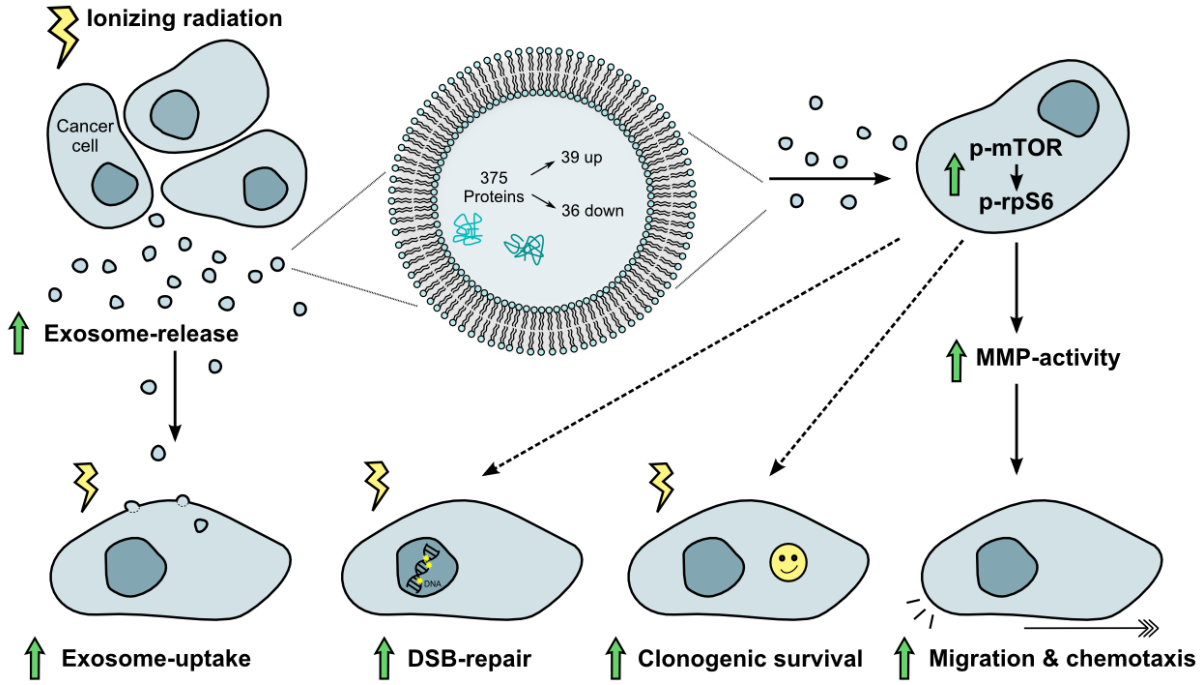

Figure 5: Effect of ionizing radiation on the exosomal communication of head and neck cancer cells. Ionizing radiation affects the exosomal release, uptake and protein composition. The transfer of exosomes isolated from irradiated HNSCC increases the level of phosphorylated mTOR and rpS6 as well as augments the activity of released MMPs. The recipient cells exhibit a faster DSB repair, increased survival, migration and chemotaxis-induced motility.

The exosomal radiation response of head and neck cancer cells is not restricted to one cell type; in fact, a multidirectional and cell type-independent interaction mechanism can be assumed. Moreover, the consequences of the exosome response from cells, such as fibroblasts, stromal cells and tumor-infiltrating immune cells within the irradiated tumor tissue, are also rarely understood. The complexity of intercellular signaling after irradiation, including the mechanistic action of exosome signaling between different cell types should be therefore further analyzed. Besides, *in vitro* exosome cross-transfer experiments, *in vivo* xenograft studies are an attractive model to understand the systemic response and the consequences of exosomes in the radiation response. Previous mechanistic studies have focused on

the role of mRNA, miRNAs, long non-coding RNAs, circular RNA and proteins in the exosome-mediated radiation response[17,18,103,225]. However, the function of other exosomal components, such as DNA, lipids, metabolites or amino acids, is not clear up to now. Future experimental approaches should therefore elucidate to what extent the individual cargo molecules contribute to the phenotypic adaptions.

In summary, exosomes increase tumor cell survival and migration processes, thus, possibly mediating radiation resistance and metastatic spread in tumor patients. Due to their pivotal role in cancer biology, exosomes might be potential biomarkers to predict and monitor treatment response of head and neck cancer patients. Based on the pro-tumorigenic action of head and neck cancer exosomes after radiation, exosome formation or uptake inhibition might counteract the exosome-mediated radioprotection. Furthermore, engineered exosomes that target specifically tumor cells and carry radiosensitizers or migration-blocking compounds might reduce the risk of radioresistance development and metastatic spread.

In conclusion, the detailed investigation of the new research topic and the underlying mechanisms of exosomes in the radiation response, the modulation of the exosomal activity and the application of engineered transport vehicles might further improve HNSCC treatment strategies.

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Figure 1: Exosome biogenesis, composition and uptake mechanisms. Multivesicular bodies (MVBs) are generated by inward budding of endosomes. To release exosomes MVBs fuse with the plasma membrane. Exosomes consist of a bilayer lipid membrane, incorporated proteins and nucleic acids. Exosomes interact with recipient cells either via surface-interaction, fusion or endocytosis. 10

Figure 2: TCGA Deregulation of signaling pathways and transcription factors. Common molecular alterations which affect cell cycle, survival, inflammation, angiogenesis, migration, differentiation and oxidative damage of HPV-negative and -positive HNSCC[133]. 17

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Figure 5: Effect of ionizing radiation on the exosomal communication of head and neck cancer cells. Ionizing radiation affects the exosomal release, uptake and protein composition. The transfer of exosomes isolated from irradiated HNSCC increases the level of phosphorylated mTOR and rpS6 as well as augments the activity of released MMPs. The recipient cells exhibit a faster DSB repair, increased survival, migration and chemotaxis-induced motility. 67

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Abbreviations

53BP1	TP53-binding protein 1
Ago2	Protein argonaute-2
Alix	Programmed cell death 6-interacting protein
AR	adaptive response
ATP	adenosine triphosphate
CD29	Integrin beta-1
CD63	CD63 antigen
CD81	CD81 antigen
CD9	CD9 antigen
CFSE	carboxyfluorescein succinimidyl diacetate ester
CTGF	Connective tissue growth factor
DDR	DNA damage response
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	double strand break
E6	Protein E6
E7	Protein E7
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-to-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinases
ESCRT	Endosomal Sorting Complex Required for Transport
FGFR1	Fibroblast growth factor receptor 1
FGFR3	Fibroblast growth factor receptor 3
Gy	Gray
HGF	hepatocyte growth factor
HIF1α	Hypoxia-inducible factor 1-alpha
hnRNPA2B1	heterogeneous nuclear ribonucleoprotein A2B1
HNSCC	Squamous head and neck carcinoma

HPV	human papillomavirus
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
ICAM-1	Intercellular adhesion molecule 1
IGFBP2	insulin-like growth factor binding protein 2
ILVs	intraluminal vesicles
KRAS	GTPase KRas
LBPA	lysobisphosphatidic acid
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MET	Hepatocyte growth factor receptor
miRNA	microRNA
MMP-13	Matrix metalloproteinase-13
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
mRNA	messenger RNA
mTOR	Serine/threonine-protein kinase mTOR
MVBs	multivesicular bodies
NO	nitric oxide
NTA	nanoparticle tracking analysis
p16-INK4a	Cyclin-dependent kinase inhibitor 2A
p53	Cellular tumor antigen p53
p70S6K	Ribosomal protein S6 kinase
PARP	Poly [ADP-ribose] polymerase 1
PBMC	peripheral blood mononuclear cell
PDK1	3-phosphoinositide-dependent protein kinase 1
PEG	polyethylene glycol
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
Ras	protein family of H-Ras, N-Ras and K-Ras
Rb	Retinoblastoma-associated protein
RIBE	radiation-induced bystander effect
RISC	RNA-induced silencing complex
RNA	ribonucleic acid

ROS	reactive oxygen species
RPS	resistive pulse sensing
rpS6	40S ribosomal protein S6
RTCA	Real-Time Cell Analyser
SDS	Sodium dodecyl sulfate
SEC	size-exclusion chromatography
SILAC	stable isotope labeling by/with amino acids in cell culture
SYNCRIP	Heterogeneous nuclear ribonucleoprotein Q
TEM	transmission electron microscopy
tRNA	transfer RNA
TSG101	Tumor susceptibility gene 101 protein
γH2AX	Histone H2AX