Insulin-Like Growth Factor-I–Mediated DNA Repair in Irradiated Salivary Glands Is Sirtuin-1 Dependent

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Abstract

Ionizing radiation is one of the most common cancer treatments; however, the treatment leads to a wide range of debilitating side effects. In patients with head and neck cancer (HNC), the surrounding normal salivary gland is extremely sensitive to therapeutic radiation, and damage to this tissue results in various oral complications and decreased quality of life (QOL). In the current study, mice treated with targeted head and neck radiation showed a significant increase in double-stranded breaks (DSB) in the DNA of parotid salivary gland cells immediately after treatment, and this remained elevated 3 h posttreatment. In contrast, mice pretreated with insulin-like growth factor-I (IGF-I) showed resolution of the same amount of initial DNA damage by 3 h posttreatment. At acute time points (30 min to 2 h), irradiated parotid glands had significantly decreased levels of the histone deacetylase Sirtuin-1 (SirT-1) which has been previously shown to function in DNA repair. Pretreatment with IGF-I increased SirT-1 protein levels and increased deacetylation of SirT-1 targets involved in DNA repair. Pharmacological inhibition of SirT-1 activity decreased the IGF-I–mediated resolution of DSB. These data suggest that IGF-I promotes DNA repair in irradiated parotid glands through the maintenance and activation of SirT-1.

Keywords: radiation, salivary glands, Sirtuin-1, DNA repair, IGF-I, xerostomia

Introduction

Each year new cases of xerostomia with a concomitant loss of salivary gland function result from radiation treatment for head and neck cancer (HNC) (Limesand et al. 2013). Radiation is a primary or secondary therapeutic modality for head and neck cancer cases and results in acute and chronic salivary gland dysfunction in most patients (Vissink et al. 2010), as well as oral complications such as xerostomia, increased dental caries, increased risk for oral infections, and malnutrition (Vissink et al. 2010). Collectively, these symptoms significantly decrease patient quality of life (QOL), and the therapeutic options for mitigating oral complications are limited by transient effectiveness, inconsistent response rates, or the induction of additional side effects (Chibly et al. 2014a).

The use of fractionated radiation timing during therapy planning is intended to allow time for normal tissues to undergo DNA repair between treatments (Hall 2000). Extensive work has described the critical molecules involved in homologous recombination (HR) and classical non-homologous end joining (C-NHEJ) pathways that repair DNA damage (Lukas et al. 2011; Ceccaldi et al. 2016). In addition, activation of these repair pathways can be mediated through the MRN complex, which consists of MRE11, NBS1, and RAD50 (Riches et al. 2008; Ceccaldi et al. 2016). The inhibition of proteins involved in these DNA damage response pathways has been incorporated in the treatment of many cancer types, including HNC (Al-Ejeh et al. 2010). Understanding the early events that regulate DNA repair in normal tissues could play a critical role in

the selection of compounds that promote tumor clearance while preventing collateral damage to normal tissues following therapeutic radiation.

The sirtuin family of proteins play a role in various cellular pathways, including energy metabolism, oxidative stress, and DNA repair (Finkel et al. 2009). These proteins were initially described to function as class III histone deacetylases and require NAD⁺ as a co-factor (Gorospe and de Cabo 2008). Sirtuin-1 (SirT-1) is one of 7 mammalian sirtuin homologues (SirT-1 to -7), and is recruited to sites of DNA damage (Finkel et al. 2009). Several proteins involved in DNA damage signaling are deacetylated by SirT-1, including p53 and Forkhead box class O (FOXO) transcription factors (Finkel et al. 2009). SirT-1 targeting of p53 and FOXO suppresses the apoptotic response and the deacetylation of NBS1 promotes DNA repair (Yuan and Seto 2007; Gorospe and de Cabo 2008).

The acute response to radiation treatment involves DNA repair, cell cycle arrest, and apoptosis pathways, and the predominance of a particular pathway is frequently tissue

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specific. Irradiated salivary glands do not induce a robust cell cycle arrest response, which leads to the induction of apoptosis and a significant loss of stimulated salivary output (Limesand et al. 2009; Mitchell et al. 2010). Treatment with insulin-like growth factor-1 (IGF-1) before radiation leads to increased G₂/M cell cycle arrest, reduced apoptosis, and complete preservation of gland secretory function (Limesand et al. 2009; Mitchell et al. 2010). This study extends these findings to show that IGF-1 promotes DNA repair via SirT-1 in salivary glands exposed to radiation. A mechanistic understanding of IGF-1-mediated DNA repair in salivary glands may significantly impact the QOL of HNC patients by alleviating the symptoms associated with radiation treatment.

Materials and Methods

Mice

All experiments were conducted in 4- to 5-wk-old FVB female mice purchased from Taconic. Mice were maintained and treated in accordance with protocols approved by the University of Arizona IACUC. The authors have completed and complied with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines/checklist for preclinical animal studies. Mice were treated with radiation, intravenous IGF-1, or a combination of the two. For irradiation, mice were anesthetized with avertin (0.4–0.6 mg/kg) and subjected to 5-Gy targeted head and neck irradiation (Theratron-80, Atomic Energy of Canada Ltd.), as previously described (Avila et al. 2009). Mice treated with IGF-1 received 5 µg of recombinant human IGF-1 (GroPrep) immediately before irradiation and mice in the irradiation group received a vehicle injection of PBS.

Neutral Comet Assay

Parotid glands were extracted at predetermined time points following radiation treatment. Glands were enzymatically dispersed using Hank's Modified Solution containing 30 mg collagenase (Roche Diagnostic Corp.) and 30 mg hyaluronidase (Sigma-Aldrich). After preparation of single-cell suspensions, the samples were centrifuged, resuspended in PBS, and diluted 1:20. Neutral comet assays were conducted as previously described (Robison et al. 2005). Images were taken with a Leica DM5500 microscope (Leica Microsystems) and a 4-megapixel Pursuit camera (Diagnostic Instruments). Approximately 25 images of individual cells were taken from each slide and analyzed using CometScore software from Freeware to generate tail moment scores.

Immunoblotting and Immunoprecipitation

Protein samples corresponding to each treatment were collected at predetermined time points. Glands were homogenized in radio immunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors, as previously described

(Limesand et al. 2006). Samples were run on polyacrylamide gels and immunoblotted, as previously described (Limesand et al. 2006). Membranes were probed with the following antibodies: histone H2A.X phosphorylated on serine (Ser)139 (Cell Signaling Technology), H2A (Cell Signaling Technology), extracellular signal-regulated kinase (ERK; Promega), replication protein A (RPA) phosphorylated on Ser4/8 (Bethyl Lab Inc.), Rad51 (Calbiochem), BRCA1 (Calbiochem), SirT-1 (H-300; Santa Cruz Biotechnology Inc.), SirT-2 (Cell Signaling Technology), SirT-3 (Cell Signaling Technology), Akt (Cell Signaling Technology). Membranes were stripped with Restore western blot stripping buffer (Thermo Fisher Scientific), reblocked, and reprobed, as previously described (Limesand et al. 2006). Densitometry analysis was conducted using ImageJ software (NIH), as previously described (Mitchell et al. 2010). Immunoprecipitation for Rad51 and BRCA1 interaction was conducted using previously described methods (Morgan-Bathke et al. 2014).

Real-time RT-PCR

Total RNA was isolated from parotid glands according to manufacturer's instructions (Qiagen). Quantitation of RNA levels by reverse transcription polymerase chain reaction (RT-PCR) was conducted, as previously described (Mitchell et al. 2010), with SirT-1 forward primer 5'-CAGACGTGGTAATGTCCA AA-3' and reverse primer 5'-TCTATGCAGGCTCTACCA CA-3' or E2F1 forward primer 5'-GGCTCTAACTGCACTTTT GG-3' and reverse primer 5'-CACCTTTGCTTATCCCCC-3'.

Immunofluorescence Staining

Paraffin-embedded tissue sections were immunostained for Rad51 or SirT-1 using previously described protocols (Chibly et al. 2014b).

Primary Parotid Acinar Cell Isolation and Treatment

Primary acinar cells were isolated from the parotid glands of FVB mice and seeded onto collagen-coated plates, as previously described (Limesand et al. 2006). After 4 d in culture, primary cells were starved overnight and irradiated. Treatment with EX527 (R&D Systems) occurred concurrently with the starvation treatment. IGF-1 treatment occurred 5 min before radiation. For the comet assay, primary cells were diluted to approximately 2×10^7 cells/ml, added to the 1% agarose solution, and treated as stated above.

Statistical Analyses

Statistical analyses were made between groups using Student's *t* tests (2-sample comparison) or ANOVA with Tukey's post hoc test (multiple comparisons) calculated with GraphPad.

Results

Resolution of Double Strand Breaks Is Enhanced in Irradiated Parotid Glands Pretreated with IGF-I

Radiation treatment can directly and indirectly (via free radicals) cause both double- and single-stranded breaks in chromosomal DNA. Double-stranded breaks (DSB) were quantified by generating tail moment scores using the neutral comet assay at predetermined time points following radiation (5-Gy dose) with or without IGF-1 pretreatment (Fig. 1A). At 15 min post-radiation, tail moment scores were 3.3 times higher than that of untreated tissues (Fig. 1A). At the same time point, pretreatment with IGF-1 resulted in tail moment scores that were not significantly different from the irradiated tissues. At subsequent time points, pretreatment with IGF-1 led to significantly reduced tail moment scores, indicating a resolution of DSB. At 3 h postradiation, tail moment scores in IGF-1 pretreated irradiated mice were similar to untreated controls, whereas irradiated mice showed tail moment scores 1.7 times higher.

Phosphorylation of the histone H2A variant H2AX at Ser139 (known as γ -H2AX) by ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) and ataxia telangiectasia and Rad3-related (ATR) occurs at sites of DNA damage and serves as a docking platform for proteins involved in DNA repair (Mah et al. 2010). In irradiated tissues, increases in γ -H2AX were observed within 5 min of treatment and remained elevated at 4 h. In mice pretreated with IGF-1, γ -H2AX also increased within 5 min of irradiation; however, at 30 min posttreatment, the levels were reduced, and this trend continued over the remaining time points. Reductions in γ -H2AX levels in mice treated with 5 Gy and IGF-1 correlated with reductions in tail moment scores. Similar levels of γ -H2AX were observed in the irradiated and IGF-1-pretreatment groups at the earliest time point (5 min), suggesting that the mice experienced a similar amount of DNA damage (Fig. 1C left side). At 30 min posttreatment, γ -H2AX levels were approximately 50% lower in mice pretreated with IGF-1 as compared with the irradiated mice (Fig. 1C right side, and 1D). These results indicate that IGF-1 promotes the resolution of DSB in salivary glands following radiation.

DNA Repair Proteins Appear Similar in Irradiated Salivary Glands with and without IGF-I Pretreatment

Because of the differences in DNA repair rates in Figure 1, the abundance and localization of various proteins involved in HR and NHEJ modes of DNA repair were initially evaluated (Fig. 2). Following radiation treatment, levels of RPA phosphorylated on Ser4/8 varied slightly but remained detectable (Fig. 2A left, 2B). Notably, pretreatment with IGF-1 did not significantly alter levels of phosphorylated RPA during the 2-h time course (Fig. 2, right), which corresponds to the time period of active DNA repair (Fig. 1A). No discernable differences in total levels of Rad51 or Ku-80 were observed in either radiation treatment group (data not shown).

Figure 1. Pretreatment with insulin-like growth factor-I (IGF-I) before radiation leads to resolution of double-stranded DNA breaks (DSB) at earlier time points as compared with radiation-only controls. The head and neck region of FVB mice was exposed to 5 Gy radiation, and the parotid salivary glands were removed at predetermined time points following irradiation. **(A)** Tissue samples were analyzed via a neutral comet assay. Average tail moment scores were determined from each parotid gland ($n = 8$ separate glands) and the data are graphed as fold over untreated controls. Asterisks denote significant differences ($P \leq 0.05$) between 5 Gy and 5 Gy plus IGF-I at each time point. **(B)** Protein samples from FVB mice were collected following exposure to 5 Gy radiation (left side) or 5 Gy radiation plus IGF-I (right side) and immunoblotted for histone H2A variant phosphorylated on Ser139 (γ -H2AX). Membranes were stripped and reimmunoblotted for total histone 2A and total extracellular signal-regulated kinase (ERK) as loading controls. **(C)** Protein samples from FVB mice treated with 5 Gy or 5 Gy plus IGF-I were collected at 5 min (left) or 30 min (right) post-treatment and immunoblotted for γ -H2AX. Membranes were stripped and reimmunoblotted for total ERK as a loading control. **(D)** Blots were analyzed by densitometry, with γ -H2AX normalized to H2A, and the data are graphed as mean intensity over untreated controls. A minimum of 4 mice per treatment group were analyzed.

Following DNA damage, Rad51 binds to ssDNA ends to form a nucleofilament, and binds to several proteins involved in DNA repair. The binding interaction between Rad51 and BRCA1 was evaluated by immunoprecipitation, and no differences were detected in either radiation group at 30 min post-treatment (Fig. 2C). In addition, increases in Rad51 nuclear foci were present in irradiated tissues when compared to unirradiated controls (Fig. 2D). These data suggest no inherent differences in the abundance and localization of DNA repair proteins between

Figure 2. Proteins involved in homologous recombination and non-homologous end joining repair appear to be similar between mice treated with radiation with or without insulin-like growth factor-1 (IGF-1). Mice were treated with targeted head and neck radiation, as described in Figure 1. **(A)** Protein samples were collected following exposure to 5 Gy (left) or 5 Gy plus IGF-1 (right) and immunoblotted for replication protein A (RPA) phosphorylated on serine (Ser)4/8. Membranes were stripped and re-immunoblotted for total extracellular signal-regulated kinase (ERK) as a loading control. **(B)** Blots were analyzed by densitometry, with phosphorylated RPA normalized to total ERK, and the data are graphed as mean intensity over untreated controls. A minimum of 4 mice per treatment group were analyzed. **(C)** Rad51 was immunoprecipitated from parotid tissue following exposure to 5 Gy or 5 Gy plus IGF-1. Membranes were immunoblotted for BRCA1 to determine binding to Rad51 (representative blot) and stripped and reimmunoblotted for total Rad51 as a loading control. **(D)** Paraffin-embedded tissue sections from parotid glands were prepared from mice untreated or treated for 30 min with 5 Gy. Slides were immunostained for Rad51 and representative images from untreated mice (left) and mice treated with 5 Gy (right) are shown. Scale bars represent 25 μ m. DAPI, 4',6-diamidino-2-phenylindole; UT, untreated.

the radiation treatment groups that could potentially account for the differences in DNA repair rates.

IGF-1 Treatment before Radiation Maintains Levels of SirT-1 and SirT-3

Since DNA repair proteins were not significantly affected in irradiated salivary glands, it was important to understand the IGF-1-mediated signaling mechanisms underlying the enhanced repair response. Previous studies have shown that IGF-1 pretreatment can promote p53 binding to cell cycle arrest genes

and suppress apoptosis in irradiated salivary glands (Limesand et al. 2009; Mitchell et al. 2010). In addition, SirT-1 activity can modulate p53-mediated apoptosis and promote DNA repair (Finkel et al. 2009). Therefore, the potential interaction of IGF-1 and sirTuins was determined. At each time point evaluated, we found that treatment with radiation decreased SirT-1 protein levels (Fig. 3A, B; Appendix Fig. 1A). In contrast, pretreatment with IGF-1 slightly increased SirT-1 protein levels when compared with unirradiated controls. Protein levels of SirT-2 were elevated following radiation exposure but there was no difference with IGF-1 pretreatment (Fig. 3A, Appendix Fig. 1B). IGF-1 treatment before radiation increased SirT-3 protein levels similar to the effect on SirT-1 but SirT-3 levels in irradiated animals were similar to that of the controls (Fig. 3A, Appendix Fig. 1C). Radiation treatment induced a 3-fold increase in SirT-1 transcripts at 30 min posttreatment, which remained elevated at 4 h posttreatment (Fig. 3C). IGF-1 pretreatment induced a similar level of SirT-1 transcripts that was not significantly different from irradiated tissues. SirT-1 protein levels in unirradiated mice treated with IGF-1 alone were not different from the untreated controls (Fig. 3D, Appendix Fig. 1D). Immunofluorescence staining of tissue sections confirmed the reduction of SirT-1 protein in irradiated glands when compared with unirradiated controls (Fig. 3E). These data suggest that IGF-1 maintains the level of SirT-1 and SirT-3 in salivary glands following DNA damage.

Pretreatment with IGF-1

Increases NBS1 Ser343 Phosphorylation and Decreases p53 Acetylation

To determine if modulating SirT-1 levels affects downstream substrates involved in DNA repair, we evaluated NBS1 phosphorylation at Ser343 and p53 acetylation. NBS1 in a hypoacetylated state permits phosphorylation at Ser343 by ATM (Yuan and Seto 2007). We found that irradiated tissues showed decreased NBS1 phosphorylation and increased p53 acetylation at 30 min posttreatment, suggesting that SirT-1 deacetylase activity is low (Fig. 4A, B). In contrast, IGF-1 treatment

before radiation promoted NBS1 phosphorylation and decreased p53 acetylation, suggesting that SirT-1 deacetylase activity is higher with IGF-1 treatment.

The E2F1 transcription factor is upregulated by DNA damage and promotes SirT-1 expression (Kwon and Ott 2008). An evaluation of E2F1 transcripts did not reveal differences in either radiation group at 30 min post-treatment (Fig. 4C). These data suggest that IGF-1 is promoting SirT-1 activity following DNA damage through mechanisms that may be distinct from mechanisms that regulate E2F1 expression.

Enhanced DNA Repair in IGF-1–Pretreated Primary Salivary Cells Is Mediated through SirT-1 Activity

To evaluate the chemical modulators of SirT-1 activation, primary acinar cell cultures were established and treated with radiation, as previously described (Limesand et al. 2006). Similar to the in vivo condition, radiation with IGF-1 pretreatment elevated SirT-1 protein levels in primary cells (Fig. 5A). The amount of DSB was determined using tail moment scores, similar to that in Figure 1A. Primary cultures treated with radiation exhibited a 2-fold increase in tail moment scores at 30 min posttreatment whereas pretreatment with IGF-1 resulted in tail moment scores similar to that of unirradiated cells (Fig. 5B). To specifically target SirT-1 activity, EX527 was used to inhibit SirT-1 deacetylase function without affecting the other sirtuins (Solomon et al. 2006). Treatment with EX527 (LD₅₀ dose, 98 nM) and IGF-1 before radiation resulted in tail moment scores that were not significantly different from that of the irradiated cells (Fig. 5B). Increasing the concentration of EX527 (150 nM and 250 nM) had a similar effect on inhibiting the resolution of DNA breaks in IGF-1 pretreated cells (Appendix Fig. 2). These data suggest that IGF-1-mediated DNA repair is dependent on SirT-1 activity.

Discussion

The activation of DNA repair pathways in normal tissues is an important consideration in the planning of fractionated radiation treatment (Hall 2000). HR and C-NHEJ are the predominant pathways facilitating DNA repair, and the MRN complex,

Figure 3. Sirtuin-1 (SirT-1) and SirT-3 are increased with insulin-like growth factor-1 (IGF-1) pretreatment as compared with radiation-only controls. Mice were treated with targeted head and neck radiation as described in Figure 1. (A) Protein samples were collected from mice treated with 5 Gy or 5 Gy plus IGF-1 at predetermined time points and immunoblotted for SirT-1, -2 or -3. Membranes were stripped and reimblotted for total extracellular signal-regulated kinase (ERK) as a loading control (representative blot). (B) Blots were analyzed by densitometry, with SirT-1 normalized to total ERK, and data are graphed as mean intensity over untreated controls. A minimum of 4 mice per treatment group were analyzed. (C) The mRNA expression level for SirT-1 was determined by quantitative RT-PCR. (D) Mice were injected with IGF-1 alone and parotid salivary glands were collected at predetermined time points. Protein samples were immunoblotted for SirT-1. Membranes were stripped and reimblotted for total ERK as a loading control. (E) Parotid tissue sections were collected 30 min after radiation treatment and immunostained for SirT-1. Representative images from untreated mice (left) and mice treated with 5 Gy (right) are shown. Scale bars represents 25 μ m. UT, untreated.

BRCA1 and ATM all function in both pathways (Ceccaldi et al. 2016). Understanding the signaling mechanisms that control DNA repair in salivary glands could lead to enhanced treatment planning to reduce collateral damage from cancer therapy. In this study, irradiated salivary glands showed a striking reduction in SirT-1 protein levels despite an increase in its mRNA expression. A similar effect on SirT-1 was found in irradiated articular chondrocytes, with a reduction in SirT-1 protein levels mediated by p38 kinase activity and proteasomal degradation (Hong et al. 2010). Regulation of SirT-1 levels appears to be controlled by transcriptional (e.g., E2F1) and posttranscriptional (e.g., HuR)

Figure 4. Pretreatment with insulin-like growth factor-1 (IGF-1) increases NBS1 serine (Ser)343 phosphorylation and decreases p53 acetylation. Mice were treated with targeted head and neck radiation as described in Figure 1. **(A)** Protein samples were collected 30 min after treatment and immunoblotted for NBS1 phosphorylation on Ser343. Membranes were stripped and reimmunoblotted for acetylated p53 and total extracellular signal-regulated kinase (ERK) as a loading control. **(B)** Blots were analyzed by densitometry, with phosphorylated NBS1 or acetylated p53 normalized to total ERK, and data are graphed as mean intensity over untreated controls. A minimum of 4 mice per treatment group were analyzed. **(C)** The mRNA expression level for E2F1 was determined by quantitative RT-PCR. UT, untreated.

processes depending on the external stimulus (Gorospe and de Cabo 2008; Kwon and Ott 2008). Reductions in SirT-1 in salivary glands correlates with inefficient resolution of DSB. In our model where secretory function is preserved using pretreatment

with IGF-1 (Limesand et al. 2009), we show that SirT-1 levels are maintained and DSB are resolved within 4 h of radiation exposure (Figs. 1 and 3). With IGF-1 pretreatment, SirT-1 can deacetylate NBS1, which subsequently promotes its phosphorylation by ATM (Fig. 4). Although other sirtuin proteins function in the DNA repair response, EX527 is highly specific for SirT-1 (Solomon et al. 2006; Finkel et al. 2009). Inhibition of SirT-1 activity by EX527 reduced the resolution of DSB (Fig. 5B), and suggests that other sirtuin proteins have little effect on the DNA repair response in salivary glands.

The signaling cross-talk between IGF-1 and SirT-1 has largely been evaluated in cell culture models, with a few in vivo reports. Prolonged IGF-1 treatment (6 d) of MCF7 cells inhibited SirT-1 activity and increased p53 acetylation resulting in high levels of senescence (Tran et al. 2014). In contrast, PI3 kinase signaling downstream from growth factor activation in C2C12 myoblast cells promotes SirT-1 retention in the nucleus and deacetylation of nuclear targets (Kwon and Ott 2008). Reducing SirT-1 levels in primary neuronal cultures results in increased survival following stress stimuli (H_2O_2 , menadione). Mechanistically, this effect was mediated through SirT-1 deacetylation of IRS-2, which led to Ras/ERK activation with no effect on Akt activation (Li et al. 2008). In skeletal muscles of mice subjected to overnight fasting followed by injection with insulin, there were higher levels of SirT-1 protein and pAkt Ser473 when compared with mice fed ad libitum and then injected with insulin. SirT-1 deacetylation of lysine residues within the PH domain of Akt and PDK1 promotes the activation of these kinases (Sundaresan et al. 2011). In the current study, the interaction between IGF-1 and SirT-1 largely appears to occur under conditions of DNA damage and not in unirradiated tissues (Fig. 3A v. 3D).

SirT-1 activity mediates a number cellular phenotypes, including aging, energy metabolism, senescence, and DNA repair. In vitro analysis suggests that increased SirT-1 activity, through overexpression or resveratrol treatment, can inhibit the induction of cellular senescence (Hong et al. 2010; Tran et al. 2014). Radiation-induced senescence has been shown in the submandibular salivary gland (Marmar et al. 2016) and we hypothesize that reductions in SirT-1 levels in vivo (Fig. 3) may act as a precursor to this phenotype. SirT-1 interacts within a network of proteins, and downstream mediators appear to be cell-type and stimulus dependent (McBurney et al. 2013). Non-histone mediators include p53, NBS1, E2F1, NF- κ B, XBP1, which play key roles in apoptosis, DNA repair, and ER stress responses that lead to cell survival (Finkel et al. 2009; Martínez-Redondo and Vaquero, 2013). Future studies aimed at deciphering which SirT-1 targets are critical in the acute response of irradiated salivary glands will hold potential for therapeutic intervention.

Following DNA damage, cell cycle arrest and DNA repair pathways are inextricably linked. DNA damage sensors, once recruited to DNA breaks, facilitate cell cycle checkpoint activation (Shaltiel et al. 2015). In addition, activation of key regulators in the cell cycle arrest response are crucial for optimal DNA repair (Satyanarayana et al. 2008; Cerqueira et al. 2009). Previous studies have shown that irradiated salivary glands are

ineffective in establishing cell cycle arrest (Mitchell et al. 2010), and this likely contributes to the slow rate of DNA repair depicted in the current study (Fig. 1). In contrast, Mitchell and colleagues discovered that IGF-1 pretreatment could induce G₂/M cell cycle arrest in irradiated salivary glands in a p53-dependent manner. The interplay between the cell cycle checkpoint and DNA damage signaling pathways induced by IGF-1 treatment likely contributed to the enhanced resolution of DSB in our study (Fig. 1).

The acute responses of the salivary glands to radiation play an integral role in establishing the chronic loss of function programs in these tissues. Following radiation treatment, significant DNA damage is detected within minutes and remains elevated 4 h posttreatment (Fig. 1). The lack of a robust cell cycle arrest response in irradiated salivary glands contributes to apoptosis, which can be detected as early as 8 h and peaks at 24 h posttreatment (Limesand et al. 2009; Mitchell et al. 2010). Collectively, the glandular responses in the first 24 h lead to significant reductions in physiological function. Interventions targeting these acute responses correlate with a benefit in salivary output. For example, activating a transient cell cycle arrest response through the use of Roscovitine can preserve salivary gland function after radiation (Martin et al. 2012). Modulating the apoptotic response through genetically engineered mouse models, disruption to PKC δ , or the administration of growth factors also preserves salivary gland function (Avila et al. 2009; Limesand et al. 2009; Arany et al. 2013; Wie et al. 2014). These studies along with the current study suggest that salivary gland radiosensitivity may be caused by an inability of the cells to activate appropriate response pathways to effectively deal with the damage.

Author Contributions

S. Meyer, contributed to conception, design, data acquisition, analysis and interpretation, drafted the manuscript; A.M. Chibly, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript; R. Burd, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript; K.H. Limesand, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Figure 5. Maintenance of sirtuin-1 (SirT-1) activity is critical for efficient DNA repair in primary salivary acinar cells. Primary acinar cells were isolated from the parotid glands of FVB mice as described in the Materials and Methods. **(A)** After 4 d in culture, cells were treated with 5 Gy or pretreated with insulin-like growth factor-1 (IGF-1) for 5 min before radiation. Cell lysates were collected 30 min after radiation and immunoblotted for SirT-1. Membranes were stripped and re-immunoblotted for total Akt as a loading control. **(B)** Primary cells were treated with 5 Gy, treated with IGF-1 before radiation (IR + IGF), pretreated with a sirtuin inhibitor (EX 527, 98 nM), or treated with IGF-1 and 98 nM of EX527 before radiation (EX + IR + IGF). Single-cell suspensions were collected 30 min after radiation and analyzed by neutral comet assay. Average tail moment scores were determined from each plate ($n = 6$) and are graphed as fold over untreated controls. Treatment groups with the same letter are not statistically different from each other. UT, untreated; IR, irradiated.

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References

- Al-Ejeh F, Kumar R, Wiegman A, Lakhani SR, Brown MP, Khanna KK. 2010. Harnessing the complexity of DNA-damage response pathways to improve cancer treatment outcomes. *Oncogene*. 29(46):6085–6098.
- Arany S, Benoit DS, Dewhurst S, Oviatt CE. 2013. Nanoparticle-mediated gene silencing confers radioprotection to salivary glands in vivo. *Mol Ther*. 21(6):1182–1194.
- Avila JL, Grundmann O, Burd R, Limesand KH. 2009. Radiation-induced salivary gland dysfunction results from p53-dependent apoptosis. *Int J Radiat Oncol Biol Phys*. 73(2):523–529.
- Ceccaldi R, Rondinelli B, D'Andrea AD. 2016. Repair pathway choices and consequences at the double-strand break. *Trends Cell Biol*. 26(1):52–64.
- Cerqueira A, Santamaria D, Martinez-Pastor B, Cuadrado M, Fernández-Capetillo O, Barbacid M. 2009. Overall Cdk activity modulates the DNA damage response in mammalian cells. *J Cell Biol*. 187(6):773–780.
- Chibly AM, Nguyen T, Limesand KH. 2014a. Palliative care for salivary gland dysfunction highlights the need for regenerative therapies: a review on radiation and salivary gland stem cells. *J Palliat Care Med*. 4(4):pii.1000180.
- Chibly AM, Querin L, Harris Z, Limesand KH. 2014b. Label-retaining cells in the adult murine salivary glands possess characteristics of adult progenitor cells. *PLoS One*. 9(9):e107893.
- Finkel T, Deng CX, Mostoslavsky R. 2009. Recent progress in the biology and physiology of sirtuins. *Nature*. 460(7255):587–591.
- Goroppe M, de Cabo R. 2008. AsSIRting the DNA damage response. *Trends Cell Biol*. 18(2):77–83.
- Hall EJ. (2000). *Radiobiology for the radiologist*. Philadelphia (PA): Lippincott Williams and Wilkins.
- Hong EH, Lee SJ, Kim JS, Lee KH, Um HD, Kim JH, Kim SJ, Kim JI, Hwang SG. 2010. Ionizing radiation induces cellular senescence of articular

- chondrocytes via negative regulation of SIRT1 by p38 kinase. *J Biol Chem.* 285(2):1283–1295.
- Kwon HS, Ott M. 2008. The ups and downs of SIRT1. *Trends Biochem Sci.* 33(11):517–525.
- Li Y, Xu W, McBurney MW, Longo VD. 2008. SirT1 inhibition reduces IGF-1/IRS-2/Ras/ERK1/2 signaling and protects neurons. *Cell Metab.* 8(1):38–48.
- Limesand KH, Chibly AM, Fribley A. 2013. Impact of targeting insulin-like growth factor signaling in head and neck cancers. *Growth Horm IGF Res.* 23(5):135–140.
- Limesand KH, Said S, Anderson SM. 2009. Suppression of radiation-induced salivary gland dysfunction by IGF-1. *PLoS One.* 4(3):e4663.
- Limesand KH, Schwertfeger KL, Anderson SM. 2006. MDM2 is required for suppression of apoptosis by activated Akt1 in salivary acinar cells. *Mol Cell Biol.* 26(23):8840–8856.
- Lukas J, Lukas C, Bartek J. 2011. More than just a focus: the chromatin response to DNA damage and its role in genome integrity maintenance. *Nat Cell Biol.* 13(10):1161–1169.
- Mah LJ, El-Osta A, Karagiannis TC. 2010. gammaH2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia.* 24(4):679–686.
- Marmary Y, Adar R, Gaska S, Wygoda A, Maly A, Cohen J, Eliashar R, Mizrahi L, Orfaig-Geva C, Baum BJ, et al. 2016. Radiation-induced loss of salivary gland function is driven by cellular senescence and prevented by IL6 modulation. *Cancer Res.* 76(5):1170–1180.
- Martin KL, Hill GA, Klein RR, Arnett DG, Burd R, Limesand KH. 2012. Prevention of radiation-induced salivary gland dysfunction utilizing a CDK inhibitor in a mouse model. *PLoS One.* 7(12):e51363.
- Martinez-Redondo P, Vaquero A. 2013. The diversity of histone versus nonhistone sirtuin substrates. *Genes Cancer.* 4(3–4):148–163.
- McBurney MW, Clark-Knowles KV, Caron AZ, Gray DA. 2013. SIRT1 is a highly networked protein that mediates the adaptation to chronic physiological stress. *Genes Cancer.* 4(3–4):125–134.
- Mitchell GC, Fillinger JL, Sittadjody S, Avila JL, Burd R, Limesand KH. 2010. IGF1 activates cell cycle arrest following irradiation by reducing binding of ΔNp63 to the p21 promoter. *Cell Death Dis.* 1:e50.
- Morgan-Bathke M, Hill GA, Harris ZI, Lin HH, Chibly AM, Klein RR, Burd R, Ann DK, Limesand KH. 2014. Autophagy correlates with maintenance of salivary gland function following radiation. *Sci Rep.* 4:5206.
- Riches LC, Lynch AM, Gooderham NJ. 2008. Early events in the mammalian response to DNA double-strand breaks. *Mutagenesis.* 23(5):331–339.
- Robison JG, Lu L, Dixon K, Bissler JJ. 2005. DNA lesion-specific co-localization of the Mre11/Rad50/Nbs1 (MRN) complex and replication protein A (RPA) to repair foci. *J Biol Chem.* 280(13):12927–12934.
- Satyanarayana A, Hilton MB, Kaldis P. 2008. p21 Inhibits Cdk1 in the absence of Cdk2 to maintain the G1/S phase DNA damage checkpoint. *Mol Biol Cell.* 19(1):65–77.
- Shaltiel IA, Krenning L, Bruinsma W, Medema RH. 2015. The same, only different - DNA damage checkpoints and their reversal throughout the cell cycle. *J Cell Sci.* 128(4):607–620.
- Solomon JM, Pasupuleti R, Xu L, McDonagh T, Curtis R, DiStefano PS, Huber LJ. 2006. Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Mol Cell Biol.* 26(1):28–38.
- Sundaresan NR, Pillai VB, Wolfgeher D, Samant S, Vasudevan P, Parekh V, Raghuraman H, Cunningham JM, Gupta M, Gupta MP. 2011. The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. *Sci Signal.* 4(182):ra46.
- Tran D, Bergholz J, Zhang H, He H, Wang Y, Zhang Y, Li Q, Kirkland JL, Xiao ZX. 2014. Insulin-like growth factor-1 regulates the SIRT1-p53 pathway in cellular senescence. *Aging Cell.* 13(4):669–678.
- Vissink A, Mitchell JB, Baum BJ, Limesand KH, Jensen SB, Fox PC, Elting LS, Langendijk JA, Coppes RP, Reyland ME. 2010. Clinical management of salivary gland hypofunction and xerostomia in head-and-neck cancer patients: successes and barriers. *Int J Radiat Oncol Biol Phys.* 78(4):983–991.
- Wie SM, Adwan TS, DeGregori J, Anderson SM, Reyland ME. 2014. Inhibiting tyrosine phosphorylation of protein kinase Cδ (PKCδ) protects the salivary gland from radiation damage. *J Biol Chem.* 289(15):10900–10908.
- Yuan Z, Seto E. 2007. A functional link between SIRT1 deacetylase and NBS1 in DNA damage response. *Cell Cycle.* 6(23):2869–2871.