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Original Article

Mesenchymal stem cell-derived HGF attenuates radiation-induced senescence in salivary glands via compensatory proliferation

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ABSTRACT

Background & Aim: Irradiation of the salivary glands during head and neck cancer treatment induces cellular senescence in response to DNA damage and contributes to radiation-induced hyposalivation by affecting the salivary gland stem/progenitor cell (SGSC) niche. Cellular senescence, such as that induced by radiation, is a state of cell-cycle arrest, accompanied by an altered pro-inflammatory secretome known as the senescence-associated secretory phenotype (SASP) with potential detrimental effects on the surrounding microenvironment. We hypothesized that the pro-regenerative properties of mesenchymal stem cells (MSCs) may attenuate cellular senescence post-irradiation. Therefore, here we evaluated the effects of adipose-derived MSCs (ADSCs) on the radiation-induced response of salivary gland organoids (SGOs).

Methods: Proteomic analyses to identify soluble mediators released by ADSCs co-cultured with SGOS revealed secretion of hepatocyte growth factor (HGF) in ADSCs, suggesting a possible role in the stem cell crosstalk. Next, the effect of recombinant HGF in the culture media of *ex vivo* grown salivary gland cells was tested in 2D monolayers and 3D organoid models.

Results: Treatment with HGF robustly increased salivary gland cell proliferation. Importantly, HGF supplementation post-irradiation enhanced proliferation at lower doses of radiation (0, 3, 7 Gy), but not at higher doses (10, 14 Gy) where most cells stained positive for senescence-associated beta-galactosidase. Furthermore, HGF had no effect on the senescence-associated secretory phenotype (SASP) of irradiated SGOs, suggesting there may be compensatory proliferation by cell-division competent cells instead of a reversal of cellular senescence after irradiation.

Conclusion: ADSCs may positively influence radiation recovery through HGF secretion and can promote the *ex vivo* expansion of salivary gland stem/progenitor cells to enhance the effects of co-transplanted SGSC.

Introduction

Head and neck cancer accounts for more than 550 000 cases annually worldwide [1] of with 70 % of patients receiving radiotherapy. A common severe side effect of radiotherapy is hyposalivation and a consequent dry mouth sensation (xerostomia) which is often very difficult to treat [2]. The major cause of this side effect is the often unavoidable exposure of the salivary glands (SG) to radiation due to their position relative to the tumor within the radiotherapy treatment plan.

SG irradiation eventually leads to a loss of acinar cells specialized in producing saliva. Resident salivary gland stem/progenitor cells (SGSCs) can proliferate in response to radiation and differentiate into acinar cells to compensate for this loss [3]. Indeed, stem/progenitor cell niches persist after irradiation but are associated with persistent DNA damage [4] and an increase in the level of cellular senescence leading to reduced stemness [5]. Cellular senescence is characterized by a stable cell-cycle arrest in response to various genotoxic, oxidative and mitochondrial

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stresses [6]. Cell senescence is accompanied by a pro-inflammatory secretome, the senescence-associated secretory phenotype (SASP), which can disrupt surrounding healthy cells through various paracrine mechanisms including paracrine senescence, inflammatory cell recruitment, fibrosis induction, and stem cell exhaustion [7]. Importantly, clearance of senescent cells by the selective removal of p16Ink4a-positive cells in genetic models, or by the senolytic drug ABT263 lead to increased stem cell self-renewal capacity and consequent increase in saliva production [5]. We hypothesize that reducing the relative abundance of senescent cells may attenuate some of the deleterious aspects associated with radiation-induced SG dysfunction [4,5].

The pro-regenerative properties of mesenchymal stem cells (MSCs) are widely documented in various contexts and tissues [8], prompting the exploration of MSC-based therapies for the SG in ongoing clinical trials [9–12]. Primary results of a Phase I/II Randomized Trial suggest MSC transplantation may ameliorate radiation-induced xerostomia, as evidenced by increased unstimulated whole salivary flow rates, and decreased symptom scores [10]. The molecular mechanisms underlying the observed improvements were not explored but are likely to be through paracrine function of transplanted MSCs. Previously described mechanisms of action are varied and include paracrine functions such as anti-apoptosis, immunomodulation, angiogenesis, anti-scarring and support of growth and differentiation of stem and progenitor cells [13]. In this study, we aimed to evaluate the secretome of adipose-derived MSCs (ADSCs) for its effect on radiation-induced senescence of the SG. For this purpose, we employed co-culture models of irradiated salivary gland organoids (SGOs) and ADSCs separated by a semi-permeable membrane, enabling the study of the influence of soluble mediators.

Materials and methods

Co-culture experiments

Primary human adipose-derived mesenchymal stem cells (ADSCs, PCS-500–011, ATCC, USA) were seeded at a density of 5000 cells/cm^2 in 12-well tissue culture grade plates and grown in MesenPro media. In parallel, salivary gland organoids (SGOs) were seeded independently in basement membrane extract (BME) domes on top of Corning® Transwell® polyester membrane cell culture inserts (pore size of 0.4 μm) and cultured in WRY media for 5 days (Fig. 1A). Organoid culture and WRY media composition is based on [14], and is described in Supplementary Materials & Methods. On day 5, when organoids had formed, SGOs were (sham)-irradiated, and transferred to 12-well plates with or without ADSCs and the co-cultures were maintained for one additional week. Following the co-culture period ADSCs were collected in RIPA buffer for protemics analysis, and SGOs were counted and/or dissociated into single cells. SGOs were then collected for RNA extraction.

Discovery-based proteomics analysis

Discovery mass spectrometric analyses were performed on a quadrupole orbitrap mass spectrometer equipped with a nano-electrospray ion source (Orbitrap Exploris 480, Thermo Scientific). The analysed samples were collected from ADSCs co-cultured alone, with SGOs, or with irradiated SGOs as described above. After 7 days of co-culture, the cell pellets of ADSCs were collected and analysed separately from SGOs cell pellets. LC-MS raw data were processed with Spectronaut (version 17.3.230224) (Biognosys) using the standard settings of the direct DIA workflow. Quantification was performed on MS1, with a human SwissProt database (https://www.uniprot.org, 20,350 entries). An extended explanation is available in Supplementary Files. Release of HGF in conditioned media was validated using a Human HGF/Hepatocyte Growth Factor ELISA Kit PicoKine® following manufacturer guidelines (Boster Biological Technology, Pleasanton CA, USA, Catalog # EK0369),

Time-lapse confluence analysis

SGOs were dissociated into single cells as described above and reseeded in uncoated 96 well plates at a density of 3000 cells/cm^2 in WRY media. The cells were allowed to adhere overnight and were next irradiated with a 137 Ce source (IBL 637 Cesium-137 γ-ray machine) using a dose rate of 0.59 Gy/min. After irradiation, additional control WRY media or WRY media supplemented with recombinant human HGF (Peprotech, cat no. 100-39H) was added up to a final concentration of 50 ng/ml based on Fig. S3. The plates were next transferred to an IncuCyte® S3 (Essen BioScience) where they were kept at 37 °C with 5 % CO2 atmosphere, and confluence was recorded every 2 h. For comparisons between treatments, the fold increase in confluence was calculated by normalizing each recorded value to the initial confluence at 0*h*00m. The area under the curve was next calculated and compared across conditions.

MTS-based proliferation assay

To measure the effect of HGF on proliferation, single cells derived from SG organoids were seeded in 96-well plates as described in the extended materials and methods at a density of 3000 cells/cm^2 in WRY media, supplemented or not with recombinant HGF (50 ng/ml). To validate receptor-ligand interaction, recombinant HGF (50 ng/ml) was added in the presence or absence of the c-MET inhibitor (GX-523, Bioconnect, The Netherlands) at a final concentration of (200 nM) in WRY media devoid of Wnt and R-spondin. At the end of the culture period (4 days post-treatment) the media was removed, and 100 μL of DMEM:F12 media and 20 μL of MTS reagent were added per well (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, The Netherlands). Cell viability was determined after 1 h of incubation by measuring the absorption at 490 nm using a microplate reader (BMG LABTECH, Utrecht, The Netherlands). Cell proliferation was calculated relative to sham untreated controls.

Results

ADSCs are a source of soluble HGF

In order to explore which proteins secreted by MSCs could potentially modulate SGSC dynamics, we first attempted to quantify the expression levels of potentially secreted proteins in ADSCs co-cultured with sham or irradiated SGOs. ADSCs and SGOs were cultured in the same well, but prevented from interacting physcially with each other by using plates with a semi-permeable membrane with a pore size of 0.4 μm, large enough to allow the transit of secreted proteins, but too small for cells to migrate through the pores. After 7 days of co-culture, ADSCs and SGOs were collected separately, and a discovery-based proteomics approach applied to ADSCs by examining samples for presence of previously established growth factors secreted by MSCs. Among the potentially secreted proteins, hepatocyte growth factor (HGF) was measured 5 to 15 times greater than several other detectable growth factors previously reported in the secretome of MSCs (Fig. 1B). To verify the secretion, we measured HGF levels in the conditioned medium (CM) of all cultures. Secretion of HGF was significantly upregulated in CM of ADSCs at 7 days post-co-culture with IR SGOs, as measured by ELISA (Fig. 1C). In addition, protein abundance of HGF correlated to higher mRNA levels, suggesting transcriptional regulation (Fig. 1D). Importantly, HGF was measured in co-cultures where ADSCs were present, but was hardly detectable when only SGOs were present, suggesting that HGF is mainly produced by ADSCs and not by the SGOs.

Probing a previously published scRNASeq dataset [15], we observed a cell-type specific production of HGF during the embryonic development of mouse SGs, where HGF is produced almost exclusively by the mesenchyme, but is completely absent in the developing epithelia, suggesting a role for HGF in SG development (Fig. 1E, Salivary Gland

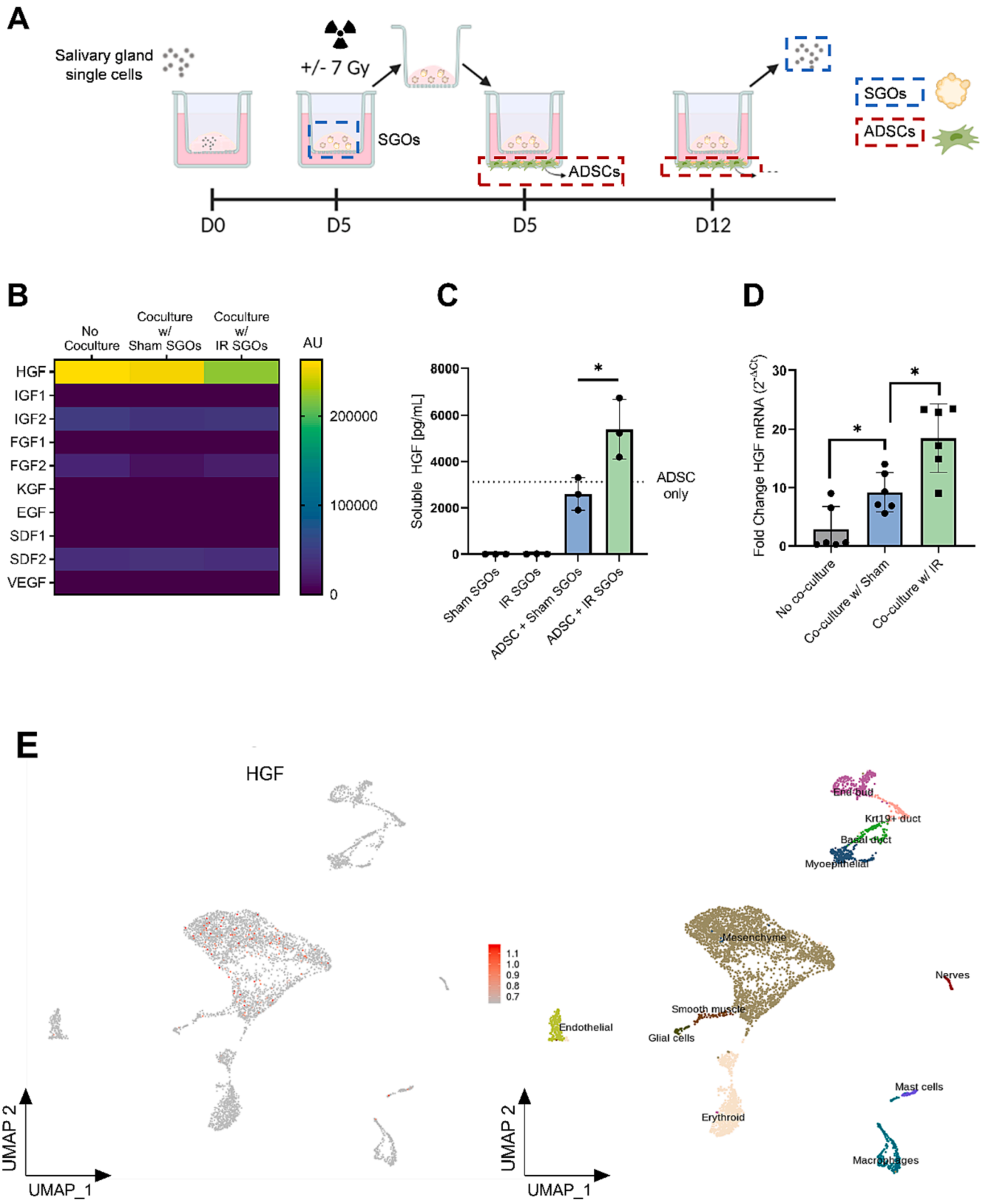

Fig. 1. Co-cultured ADSCs with salivary gland organoids are a source of soluble HGF. (A) Adipose-derived mesenchymal stem cells (ADSCs) co-cultured with salivary gland organoids (SGOs) were subjected to a discovery-based proteomics approach, (B) revealing increased production of HGF in relation to other growth factors (pooled data from n = 3). (B) Secretion of HGF was confirmed at the protein level via ELISA in the conditioned media of ADSCs at 7 days post-co-culture with IR SGOs (*$p < 0.001$, in two-way ANOVA, n = 3). (D) HGF mRNA levels were measured post-co-culture by RT-qPCR (*$p < 0.01$, unpaired *t*-test, n = 6). (E) Single cell RNA-seq presents mesenchyme enriched HGF transcripts in mouse embryonic salivary glands (NIDCR, SGGEA).

Gene Expression Atlas).

Adult salivary gland stem progenitor cells remain responsive to HGF stimulation

HGF expression in the salivary gland is mostly lost after embryonic development, however, the expression of its non-promiscuous receptor cMET persists in adult tissues. We hypothesized HGF may have a physiological role upon injury in adult tissues, as has been described for the liver, where HGF orchestrates liver regeneration after partial hepatectomy [16]. We decided to test the effect of HGF on adult SGSCs, with a potential view to optimizing their regenerative properties for transplantation purposes in radiation-induced xerostomia patients. To test this, SG cells were isolated from the submandibular gland of C57BL/6 mice and expanded *ex vivo* to promote the continued self-renewal [14].

SG cells were seeded as single cells to allow the formation of a cellular monolayer. The addition of recombinant HGF to the culture media had a positive influence on the proliferation of SG cells, as evidenced by a significant increase in confluency (Fig. 2A-B), and cell proliferation (Fig. 2C). Importantly, SG cells irradiated with a total dose of 7 Gy also responded to HGF stimulation, albeit to a lesser extent than sham-irradiated cells (Fig. 2C). These findings suggest irradiated SG cells retained some proliferative capacity after a 7 Gy dose of radiation and were also able to respond to HGF supplementation. Importantly, the effect of HGF appeared to be mediated by its canonical receptor cMET, as pharmacological inhibition of cMET with the small molecule GX-528, or a neutralizing anti-HGF antibody (Fig. S1), fully abolished the effect of HGF on increased proliferation (Fig. 2D).

SG cells grown as monolayers are unable to form complex structures, which are required to generate the increased surface areas necessary for producing adequate amounts of saliva [17]. Therefore, we decided to test the effect of HGF on our previously developed SGO model (Fig. 2E), which contain SG stem progenitor cells that can give rise to all major SG cell types [14,18]. Addition of HGF to SGO cultures resulted in a higher number of cells (mean fold change: 1,62 ± 0,09) per organoid (Fig. 2F), suggestive of increased proliferation. Similarly, a positive effect on proliferation was observed in 7 Gy irradiated cells (mean fold change: 1,10 ± 0,11) (Fig. 2F). However, when the SGOs were re-seeded after HGF treatment to form new organoids as a measure of stem cell capacity, no extended effect was observed in HGF treated cells (Fig. 2G),

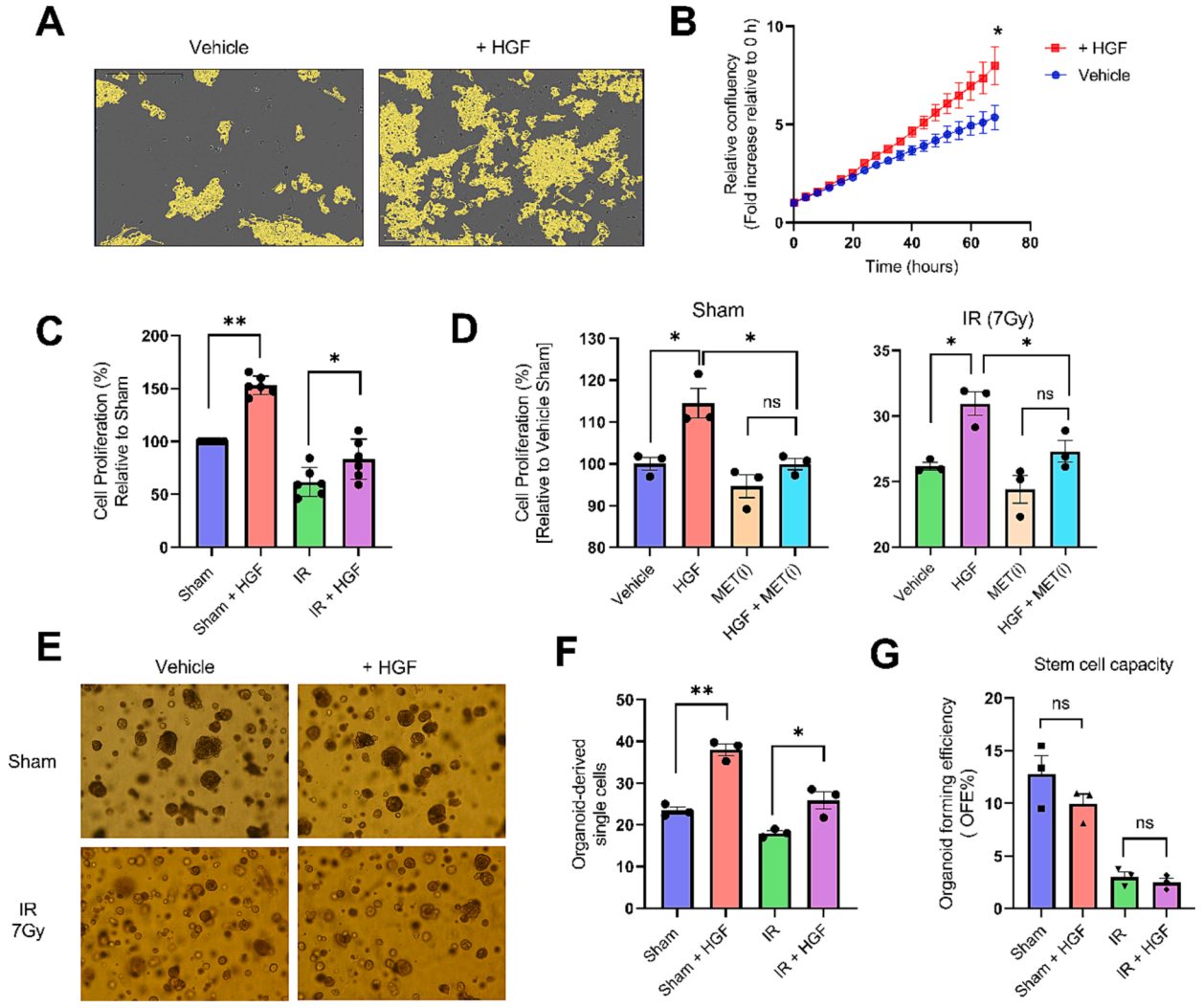

Fig. 2. HGF stimulates the proliferation of ex vivo cultured salivary gland cells. (A) Salivary gland cells were seeded as monolayers and treated with vehicle or recombinant HGF. (B) Confluency was monitored over time and the relative increase was calculated in relation to the initial confluency (mean + SEM, n = 6, *p < 0.01). (C) Cell proliferation was estimated based on metabolic activity in sham or irradiated cells (**p < 0.01, * p < 0.05, in unpaired *t*-test, n = 3), and (D) under pharmacological inhibition of the HGF receptor cMET (unpaired *t*-test, n = 6). (E) Sham-irradiated salivary gland organoids were treated with vehicle or recombinant HGF. After 7 days in culture, organoids were dissociated, and (F) cell number was calculated to estimate proliferation. (G) Organoid-derived single cells were re-seeded at equal densities and cultured in the absence of HGF for 7 additional days to estimate stem cell capacity based on organoid forming efficiency (**p < 0.01, * p < 0.05, ns: not significant, unpaired t-tests, n = 3) [24].

suggesting the pro-proliferative effect is only exerted when HGF is present.

HGF attenuates radiation-induced premature senescence of salivary gland cells

We have previously shown radiation-induced cellular senescence of SG stem cells plays a role in the permanent hypofunction of irradiated SGs [5]. Therefore, we decided to study the effect of HGF supplementation on radiation-induced cellular senescence. For this, we established a radiation dose–response on SG cells using doses of 3, 7, 10 and 14 Gy. As expected, growth arrest was observed with increasing doses of radiation, reaching a plateau at radiation doses higher than 10 Gy (Fig. 3A-B). Importantly, supplementation with HGF resulted in increased proliferation of sham-irradiated cells, and of cells exposed to lower doses of radiation (3 Gy, 7 Gy) (Fig. 3A-B). However, no effect was observed at higher doses of radiation (10, 14 Gy), suggesting these cells although viable were proliferation incompetent and more likely to be senescent. Senescence-associated beta-galactosidase (SA-β-gal) staining confirmed the induction of senescence after irradiation in both 2D monolayers (Fig. 3C, Fig. S2), and 3D organoids (Fig. 3D). The majority of cells stained positive at 7 Gy and supplementation with HGF only had a modest but significant effect on SA-β-gal staining, implying that the majority of the cells remained senescent (Fig. 3C,D). Furthermore, HGF supplementation stimulated DNA synthesis, as evidenced by an increase of cells in the S-phase of the cell cycle (Fig. 3D). However, HGF treatment did not have a significant effect on selected transcripts of the SASP (Fig. 3E). These findings suggest there was no reversal of cellular senescence upon HGF treatment. Instead, we propose HGF supplementation stimulates cell division in surviving, proliferation competent SGSCs that are not fully senescent.

HGF supplementation stimulates ex vivo expansion of patient-derived salivary gland cells

Head and neck cancer patients whose SGs have been irradiated may have reduced numbers of SG stem progenitor cells, posing difficulties for the *ex vivo* expansion of transplantable cells. In humans, data from the salivary gland gene expression atlas suggests HGF and MET expression is upregulated in response to ionizing radiation (Fig. 4A), suggesting a similar mechanism of HGF-dependent compensatory proliferation may be in place. We therefore decided to investigate if patient-derived salivary gland cells may respond similarly to HGF stimulation after irradiation. Salivary gland cell monolayers responded to HGF with increased confluency (Fig. 4B). Moreover, an increased proliferation of sham and 7 Gy irradiated SGOs was observed (Fig. S4), which were able to form more organoids after 7 days of continuous HGF stimulation (Fig. 4C, D). These findings suggest patient-derived SGOs remain responsive to HGF stimulation after radiation, and may also benefit from HGF supplementation for *ex vivo* expansion for transplantation purposes.

Discussion

Radiation-induced hyposalivation and consequent xerostomia remains a clinically relevant problem that severely hampers patients' quality of life. Currently, there are no available treatments that effectively tackle the underlying cause, and patients instead rely on temporary symptom management (e.g., sialagogues). Recent strategies to rescue saliva production, instead of managing its symptoms, suggest the autologous transplantation of adult SGPCs may be a viable option [19]. However, irradiation in patients prior to *ex vivo* expansion may result in a reduced quality of SGPCs with a subset of these presenting persistent DNA damage, and consequent cellular senescence [4,5]. Since SGPCs are already in use in a clinical trial for treatment of radiation-induced hyposalivation, the need to dissect exploitable mechanisms to develop strategies for expanding remaining stem cells *in situ* or *ex vivo* is clinically relevant.

In this study, we probed the interplay between adipose-derived cells of mesenchymal origin and salivary gland organoids composed mostly of epithelial cell types. In doing so, we confirmed the role of HGF as a mesenchymal-derived growth factor with mitogenic effects on both murine and human adult SG cells, a role previously described in branching morphogenesis of the SG during embryonic development [20,21]. HGF was first identified in the hunt for a phantom factor that orchestrates liver regeneration after partial (70 %) hepatectomy [16], following the recognition of the extraordinary regeneration capacity of the liver, which is maintained well into adulthood. The increase of HGF transcripts in the SG after irradiation, and the ability of adult SG cells to be stimulated by HGF suggests analogous regeneration programs may also be present in damaged SGs.

Here, HGF supplementation was unable to stimulate cell proliferation at high doses of radiation (>10 Gy). These data are in agreement with our earlier findings that growth factors are only able to improve SG function below a certain dose of radiation beyond which no viable stem cells remain [22]. Many of these cells following 10 Gy radiation remain metabolically viable and express SA-β-gal, indicative of cellular senescence. Put together, these findings suggest HGF is unable to revert radiation-induced senescence, and instead stimulates cell division in proliferation-competent cells. This may be a preferable outcome, as it would avoid promoting the proliferation of DNA-damaged cells that may accumulate further mutations thereby increasing the possibility of malignant transformation.

Soon after its discovery, dysregulated HGF signalling was implicated in oncogenic contexts as a key tumor-promoting factor in the tumor microenvironment (reviewed in [23]), highlighting caution that must be exercised when employing HGF treatment in cancer patients. In this study, the increased cell proliferation observed with HGF stimulation ceased after HGF was removed from the culture media, suggestive of no malignant transformation. However, a comprehensive characterization of the effects of HGF on the long-term expansion of transplantable stem cells would be desirable. Considering the latter, it may not be wise to use HGF as an *in vivo* proliferation stimulator but could be used to expand donor cells *ex vivo* prior to transplantation. Alternatively, as MSCs can be locally injected and secrete their cargo locally, this may represent an interesting approach to stimulate cells that only receive a low dose of RT [19]. Furthermore, HGF was unable to dampen the pro-inflammatory SASP of irradiated cells. Due to the deleterious effects that the SASP may have on the stem cell niche, future strategies relying on *in situ* supplementation of HGF may also benefit from the concomitant elimination of senescent cells via senolytics. Given the complexity of living tissues, and the possible involvement of immune cell-types, which are lacking in our co-culture models, the use of *in vivo* experiments may provide additional evidence for the effectiveness of this strategy.

In conclusion, ADSC-promote the *ex vivo* expansion of SGSCs via HGF secretion and may enhance the effects of co-transplanted SGSC in radiation-induced xerostomia patients.

CRediT authorship contribution statement

A. Soto-Gamez: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft, Project administration. **M. van Es:** Investigation. **E. Hageman:** Investigation. **S.A. Serna-Salas:** Resources. **H. Moshage:** Resources. **M. Demaria:** Supervision, Writing – review & editing, Funding acquisition. **S. Pringle:** Supervision, Writing – review & editing, Funding acquisition. **R.P. Coppes:** Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Coppes R.P. reports financial support was provided by Dutch Cancer

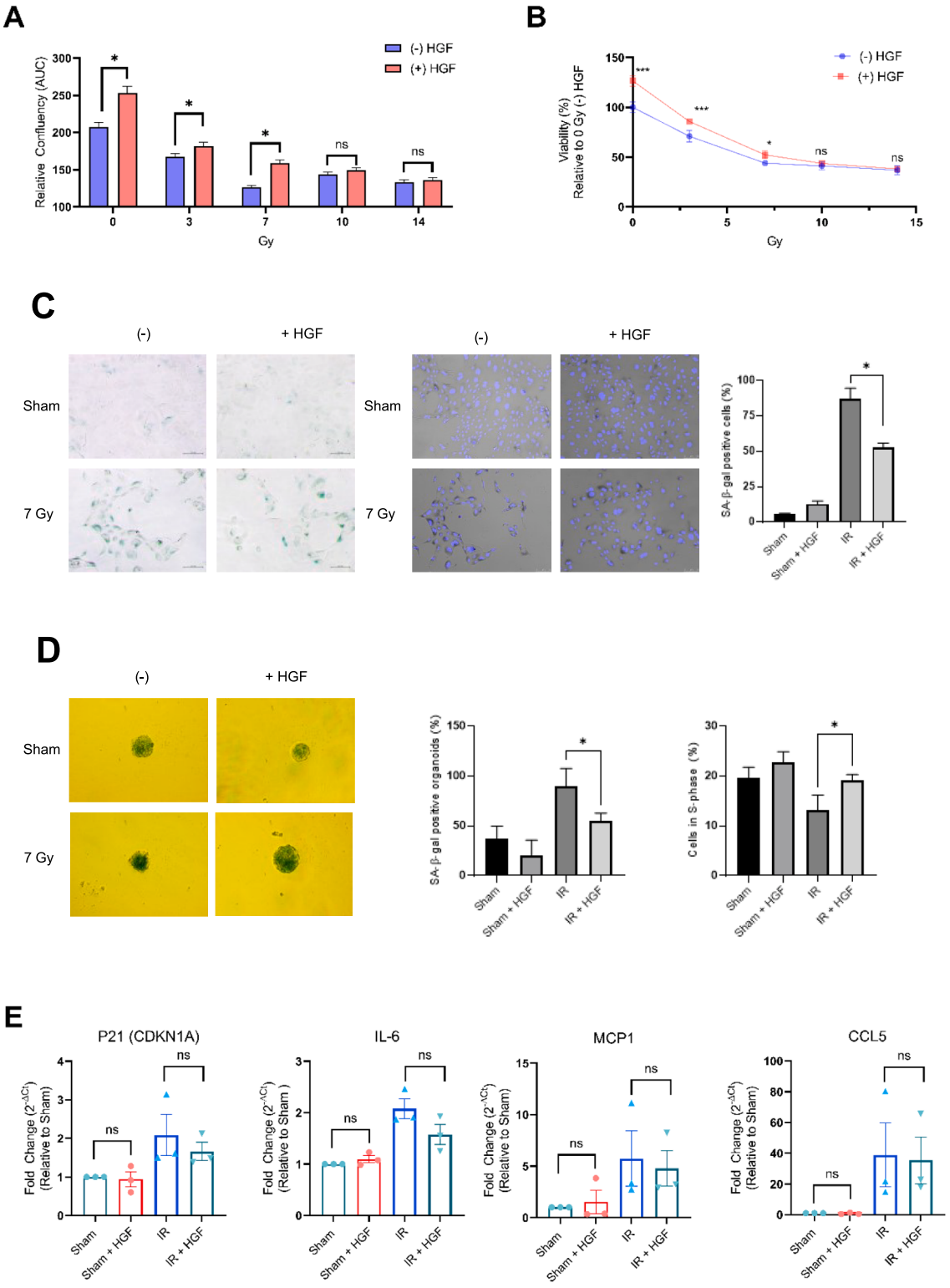

Fig. 3. HGF attenuates radiation-induced premature senescence. (A) Salivary gland cells were subjected to escalating doses of radiation and cultured in the presence or absence of HGF to determine effects on confluency, (B) cell proliferation, and (C) senescence-associated beta-galactosidase. (D) Salivary gland organoids (SGOs) were irradiated and supplemented with HGF (50 ng/mL) for seven additional days after which organoids were stained for senescence-associated beta-galactosidase, the percentage of positive organoids was estimated, and the percentage of cells in the S-phase was calculated based on flow cytometric cell cycle analysis using a Watson (Pragmatic) model [25]. (E) Senescent cell mRNA marker expression was measured (mean + SEM are shown, * $p < 0.01$, unpaired t-tests, n = 3).

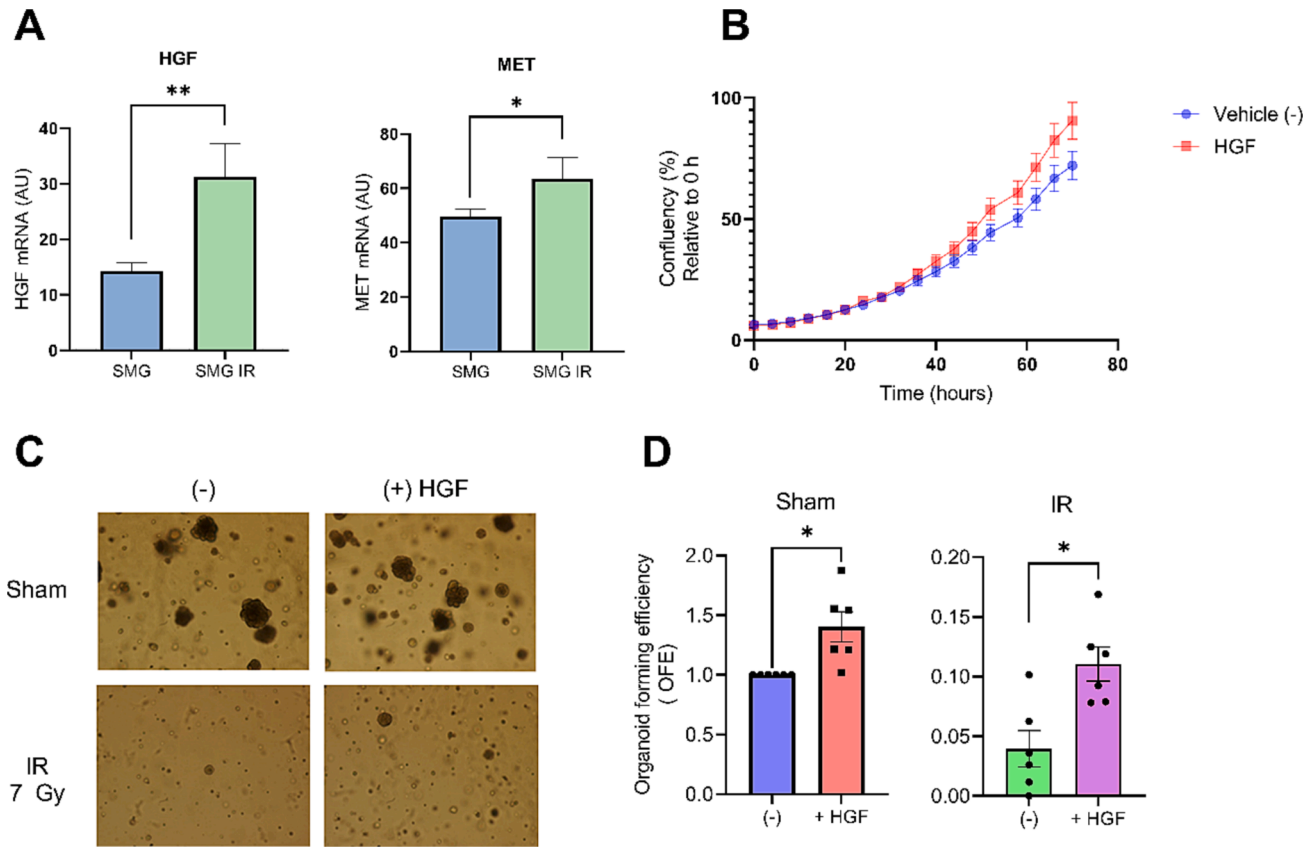

Fig. 4. HGF stimulates the proliferation of patient-derived salivary gland organoids after irradiation. (A) HGF and MET expression in bulk RNA-seq from patient-derived whole submandibular glands ([13,26], SGGEA). (B) Patient-derived salivary gland cells were grown in monolayers and supplemented with HGF (50 ng/ml), confluency was calculated relative to the initial density (mean + SEM, *p < 0.05, unpaired *t*-test, n = 3). (C) Single cells were seeded and (sham)-irradiated for 3D organoid formation, cells were treated with HGF (50 ng/ml) or vehicle and (D) the organoid forming efficiency after 7 days of stimulation was calculated relative to vehicle (-) treated sham controls (mean + SEM, * p < 0.05, unpaired *t*-test, n = 6).

Society. Coppes R.P. serves as an editor for Radiother Oncol.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2023.109984.

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