Cell Extracts Functionally Restore Irradiation-injured Salivary Glands

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January, 2017

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy in Dental Science

CONTENTS

ABSTRACT

Salivary hypofunction is a major side effect of radiotherapy for patients with head and neck cancer. Our group used a cell extract from whole bone marrow (BM) cells, named BM Soup, which contains numerous cytokines, growth factors, and other paracrine factors, to rescue the function of irradiation (IR)-injured salivary glands (SGs). The results demonstrated that BM Soup restored the salivary flow rate, protected salivary cells from IR damage, and upregulated the expression of genes related to tissue repair/ regeneration. However, the components of BM Soup which are responsible for the therapeutic effects remain unknown. The first aim of this thesis was to demonstrate that proteins are the active ingredients. We devised a method using proteinase K followed by heating to deactivate proteins and for safe injections into mice. BM Soup and "deactivated BM Soup" were injected into mice that had their salivary glands injured with 15Gy IR. Results at week 8 post-IR showed the 'deactivated BM Soup' was no better than injections of saline, while injections of native BM Soup restored saliva flow, protected salivary cells and blood vessels from IR-damage. Protein arrays detected several angiogenesis-related factors (CD26, FGF, HGF, MMP-8, MMP-9, OPN, PF4, and SDF-1) and cytokines (IL-1ra, IL-16) in BM Soup. In conclusion, the native proteins are the bioactive ingredients in BM Soup for functional salivary restoration following IR.

For any given therapy, the frequency and timing of treatment are the important parameters in the restoration of salivary glands. The second aim of this thesis was to optimize the frequency and timing of BM Soup injection in mice with IR-damaged SGs. The results showed that BM Soup injections initiated between 1-3 weeks mitigated the IR-induced injury to SGs, while delayed treatment had no saliva secretion improvement. In addition,

although the therapeutic effect of BM Soup lessened after 8 weeks, it can be sustained by increasing the frequency of weekly injections.

BM cell harvesting remains an invasive procedure and can lead to donor discomfort and in severe cases, to life-threatening complications, such as cardiopulmonary problems, bacterial infections, and cerebrovascular accidents. The third aim of this thesis was to test if other types of tissues, which were either clinically easier to harvest (such as the adipose tissue) or regarded as 'dispensable organ' (such as the spleen), could be used as alternate sources of "Cell Soup" for the repair of IR-injured salivary glands. The results demonstrated that both Adipose-derived stromal cell Soup and Spleen Soup showed comparable therapeutic effects with BM Soup at eight weeks post-IR, but BM Soup and Spleen Soup maintained the therapeutic effects for a longer follow-up time (16 weeks).

In summary, BM Soup can mitigate ionizing radiation injury to salivary glands. The native protein components are the bioactive ingredients for this therapeutic effect. Starting injections of BM Soup within three weeks post-IR improves the restoration of salivary glands, and the effect can be sustained by increasing the frequency of weekly injections. In addition, alternate "Cell Soups", such as ADSC Soup and Spleen Soup, have comparable therapeutic effects with BM Soup. This molecular therapy approach has great potential for future clinical application.

RÉSUMÉ

L'hypofonction salivaire est un effet secondaire majeur de la radiothérapie pour les patients atteints des cancers de la tête et du cou. Pour rétablir la fonction des glandes salivaires lésées par irradiation (IR) notre groupe utilise un extrait cellulaire des cellules de la moelle osseuse (BM, pour *Bone Marrow*), nommée Soupe BM qui contient de nombreuses cytokines, facteurs de croissance et autres facteurs paracrine. Les résultats ont démontré que la soupe BM rétablit le débit salivaire, protège les cellules salivaires des lésions de l'IR et augmente l'expression des gènes liés à la réparation et la régénération des tissus. Cependant, les composantes de la soupe BM qui sont responsables des effets thérapeutiques restent inconnues. Le premier objectif de cette thèse est de démontrer que les protéines sont les ingrédients actifs dans ce processus thérapeutique. Nous avons mis au point une méthode utilisant la protéinase K suivie d'un chauffage pour désactiver les protéines, ainsi que pour assurer la sécurité des injections dans la souris. La soupe BM et la « soupe BM désactivée » sont injectées dans les souris qui ont leurs glandes salivaires lésées avec 15Gy d'IR. Huit semaines après l'IR, les résultats montrent que la « soupe BM désactivée » n'est pas meilleure que les injections de saline (contrôle négatif), alors que les injections de soupe BM rétablissent le flux salivaire, protègent les cellules salivaires et les vaisseaux sanguins contre les lésions IR. Avec un immunobavardage de style « array » commercial, les anticorps sur la membrane ont détecté plusieurs facteurs liés à l'angiogenèse (CD26, FGF, HGF, MMP-8, MMP-9, OPN, PF4 et SDF-1) et des cytokines (IL-1ra, IL-16) dans la soupe BM. En conclusion d'après nos résultats les protéines sont les ingrédients bioactifs dans la soupe BM pour la restauration salivaire après IR.

Pour toute thérapie, l'initiation et la fréquence du traitement sont des paramètres importants dans la restauration des glandes salivaires. Le deuxième objectif de cette thèse est d'optimiser le début et la fréquence d'injection de soupe BM chez la souris avec des glandes salivaires endommagées par IR. Les résultats montrent que les injections de soupe BM initiées entre 1 et 3 semaines atténuent la lésion induite par IR aux glandes salivaires, alors que retarder le traitement mène à une absence d'amélioration de la sécrétion salivaire. De plus, bien que l'effet thérapeutique de la soupe BM diminue après 8 semaines, il peut être maintenu en augmentant la fréquence des injections hebdomadaires.

La collecte de cellules BM reste une procédure invasive et peut mener à l'inconfort du donneur et, dans des cas graves, à des complications potentiellement mortelles, telles que des problèmes cardiopulmonaires, des infections bactériennes et des accidents vasculaires cérébraux. Le troisième objectif de cette thèse est de tester si d'autres types de tissus, cliniquement plus faciles d'accès (tels que le tissu adipeux) ou considérés comme des « organes dispensables » (tels que la rate) peuvent être utilisés comme des sources alternatives à la moelle osseuse pour la réparation des glandes salivaires blessées. À huit semaines après l'IR, les résultats démontrent que les cellules stromales dérivées du tissu adipeux (ADSC) et les cellules de la rate possèdent des effets thérapeutiques comparables avec la soupe BM. Cependant, seulement la soupe BM et la soupe de la rate maintiennent leurs effets thérapeutiques pour une durée de suivi plus long (16 semaines) que la soupe ADSC.

En conclusion, suivant un traitement d'IR, la soupe BM peut atténuer les dommages des glandes salivaires. Les composants protéiques naturels sont les ingrédients bioactifs qui produisent l'effet thérapeutique. Le début des injections de soupe BM dans les trois

semaines suivant IR améliore la restauration des glandes salivaires, et l'effet peut être maintenu en augmentant la fréquence des injections hebdomadaires. De plus, les soupes cellulaires alternatives, telles que la soupe ADSC et la soupe de la rate, ont des effets thérapeutiques comparables avec la soupe BM. Cette approche de thérapie moléculaire a un grand potentiel pour une future application clinique.

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere and deepest gratitude to my supervisor, Dr. Simon Tran, for his encouragement, motivation, patience, and continuous support. It has been a great honor to be his student, and to study in his laboratory team has been an amazing experience. Whenever I need help with my research or writing, his office is always open. Without his precious guidance, I could not have achieved this endeavor.

Besides my supervisor, I would like to express my sincere gratitude to Dr. Jan Seuntjens for his help in animal irradiation. I also want to thank my Ph.D. committee members, Dr. Jan Seuntjens, Dr. Dieter Reinhardt, and Dr. Svetlana Komarova, for their expertise, time, and invaluable comments on my research projects.

My sincere thanks also goes to Dr. Younan Liu for his suggestion and assistance in designing and performing the experiments. I am profoundly grateful to him for teaching me the fundamentals of research

I would like to thank my collaborators, Dr. Shen Hu, Dr. Faleh Tamimi, Dr. Marta Cerruti, Dr. Xingyi Xie, Dr. Mohamed-Nur Abdallah, and Dr. Michael Roskies for their time and insightful advice.

I would like to thank Kaartinen lab, Komarova lab, Tamimi lab, McKee lab, Bui lab, FEMR, and Center for Bone and Periodontal Research for sharing their resources.

Many thanks to all Tran research group members for their kind help with my experiments and writing. Their invaluable assistance was critical in helping me finish my manuscript. A big thank you to Li-Chieh Lin and André Charbonneau for the French translation of the

thesis abstract. I would also like to thank all my friends with whom I went through successes and challenges, and shared laughs and hardships.

Last but not least, I would like to express my sincere gratefulness to my wife, my kids, my parents, and all my family members in China, who have supported me throughout my Ph.D. studies. You raise me up to more than I can be. I love you all.

CONTRIBUTIONS OF AUTHORS

This dissertation includes two published papers and an unpublished manuscript of which the doctoral candidate is the first author. Contributions of all authors are provided as below:

1. Identification of the active components in Bone Marrow Soup: a mitigator against irradiation-injury to salivary glands.

Dongdong Fang, Shen Hu, Younan Liu, Vu-Hung Quan, Jan Seuntjens, and Simon D Tran

The candidate designed and performed all experiments and data analysis, and wrote the manuscript.

Shen Hu assisted in experiment design and proof-read the manuscript.

Younan Liu assisted in designing and performing experiments.

Vi-Hung Quan assisted in designing experiments.

Jan Seuntjens conducted the animal irradiation and assisted in designing experiment and proof-read the manuscript.

Simon Tran designed and supervised the whole study, and wrote and final approved the manuscript.

2. Optimal timing and frequency of Bone Marrow Soup therapy for functional restoration of salivary gland injured by single dose or fractionated irradiation

Dongdong Fang, Sixia Shang, Younan Liu, Mohammed Bakkar, Yoshinori Sumita, Jan Seuntjens, Simon D Tran

The candidate designed and performed all experiments and data analysis, and wrote the manuscript.

Sixia Shang performed the experiments and data analysis.

Younan Liu assisted in designing and performing experiments.

Mohammed Bakkar assisted in animal irradiation.

Yoshinori Sumita assisted in designing experiments and proof-read manuscript.

Jan Seuntjens conducted the animal irradiation and assisted in designing experiment and proof-read the manuscript.

Simon Tran designed and supervised the whole study, and wrote and final approved the manuscript.

3. Therapeutic effects of cell soups from different tissues on restoration of irradiation-injured salivary glands

Dongdong Fang, Younan Liu, JinChoon Lee, Jan Seuntjens, Simon D Tran

The candidate designed and performed all experiments and data analysis, and wrote the manuscript.

Younan Liu assisted in designing and performing experiments.

JinChoon Lee assisted in culturing adipose-derived stromal cells.

Jan Seuntjens conducted the animal irradiation and assisted in designing experiment and proof-read the manuscript.

Simon Tran designed and supervised the whole study, and wrote and final approved the manuscript.

ABBREVIATIONS

Ach	Acetylcholine
ADSC	Adipose-derived stromal cell
Ang-3	Angiopoietin-3
BM	Bone marrow
BMP7	Bone morphogenetic protein 7
BMS	Bone marrow soup
CFA	Complete Freund's adjuvant
DAPI	4,6-diamidino-2-phenylindole, dihydrochloride
DFP	Diisopropyl fluorophosphates
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFRα2	GDNF family receptor alpha-2
HGF	Hepatocyte growth factor
IGF-1	Insulin growth factor-1
IL-1, -16	Interleukin-1, -16
IMRT	Intensity-modulated radiotherapy
IR	Irradiation
Keratin 5	CK 5
KGF	Keratinocyte growth factor
MMP	Matrix metalloproteinase
MSC	Mesenchymal stromal cell
NGF	Nerve growth factor

OPN	Osteopontin
PAI-1	Plasminogen activator inhibitor-1
PCNA	Proliferating cell nuclear antigen
PF4	Platelet factor 4
PK	Proteinase K
PMSF	Phenylmethylsulfonyl fluoride
PTX3	Pentraxin 3
SDF-1	Stromal cell-derived factor 1
SFR	Salivary flow rate
SG	Salivary gland
TDT	Terminal deoxynucleotidyl transferase
TF	Transfer factor
TGFb1	Transforming growth factor beta 1
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
α -SMA	alpha-smooth muscle actin

Chapter 1 - Introduction

Salivary glands and saliva

Human salivary gland system consists of two exocrine groups; the three paired major glands including the parotid, submandibular, and sublingual glands, and numerous minor glands distributed throughout the oral cavity [1]. In gross anatomy, the parotid glands are the largest of the major salivary glands, followed by submandibular glands, while the sublingual glands are the smallest. All major salivary glands have a similar anatomic structure, including secretory acinus and ductal system. Acinus has two types of acinar cells, the serous cells and the mucous cells， and is the only site to secrete fluid and provide ~85% of the secreted proteins in saliva[2]. In human, parotid glands contain pure serous acinar cells, and submandibular glands contain both with serous cells predominating, while the majority of acinar cells in sublingual glands are mucous. The salivary ductal system is composed of three segments, the intercalated, striated (intralobular), and excretory (extralobular) ducts. The intercalated duct is formed by cuboidal epithelium. As the diameter of duct increased, the epithelial lining is changed to stratified columnar, then to the non-keratinized squamous cells[3]. Salivary ductal cells are water impermeable, but can secrete salivary proteins and reabsorb NaCl from saliva.

Saliva secretion is the primary function of salivary glands. Fluid secretion is a two-stage process: 1) acinar cells secrete the NaCl-rich primary saliva to the lumen; 2) primary saliva is modified during flowing through the salivary ducts[4]. The movement of water in acinar cells is driven by the osmotic gradient. Cl^- is transported and concentrated in the acinar cells through basolateral Na^+-K^+-$2Cl^-$ cotransporter, and efflux across the apical membrane to the lumen by the Ach-evoked Ca^{2+}-dependent Cl^- pathway. Then, an osmotic gradient is

established to move the water from the capillaries to acinar cells, then the lumen. With the assistance of myoepithelial cells, the fluid is expelled into salivary ducts[5]. During the passage through the ductal tree, the ionic composition of saliva is modified. NaCl is reabsorbed by both intra and extralobular ductal epithelium. Specifically, Na^+ is absorbed by the ENaC, an epithelial Na^+ channel, and a Na^+/H^+ exchanger in the apical membrane of ductal cells, while the reabsorption of Cl^- involves apical Cl^- channels and Cl^-/HCO_3^- exchangers. Meanwhile, $KHCO_3$ is also secreted into the fluid by apical K^+/H^+ exchangers and K^+-HCO_3^- cotransporters[6]. The ironic component modification of fluid results in a hypotonic saliva. Protein components in saliva are synthesized in the rough endoplasmic reticulum, then transported, through Golgi complex, condensing vacuoles, and secretory granules, to the apical membrane. Then, protein contents are released into the lumen via exocytosis[7]. The secretion of saliva is almost completely regulated by both autonomic nerves, parasympathetic and sympathetic nerves [8]. Parasympathetic nerves play a critical role in saliva fluid secretion from acinar cells. Acetylcholine (ACh), a neurotransmitter secreted by parasympathetic nerves, interacts with M3 and M1 receptors of salivary glands, and then increases the intracellular Ca^{2+} concentration, which leads to the salivary fluid secretion[9,10]. Sympathetic nerves mainly contribute to the protein secretion from both acinar cells and ductal cells. Norepinephrine secreted by sympathetic nerves activates β-adrenergic receptor resulting in the formation of intracellular cAMP. It causes the activation of protein kinase A and phosphorylation of proteins, which further leads to the protein secretion.

Saliva plays a critical role in the process of food digestion and oral hygiene maintenance. Specifically, A-amylase in saliva, named ptyalin, can break down the starches into maltose,

maltotriose, and α-limit dextrin. The serous glands of the tongue (Ebner) secrete lingual lipase, which initially breaks down the lipid in the acidic stomach[3]. Moreover, the mucus components in saliva can facilitate the formation of food boluses and ease the processes of chewing and swallowing. In addition, saliva serves as a medium to dissolve the tastants and present to the receptors of taste cells. Saliva has lubricatory and protective functions. Mucins secreted by minor salivary glands coats the surface of oral mucosa due to its rheological properties including low solubility, high viscosity and elasticity, serving as a barrier to prevent desiccation and protect the soft tissues from the mechanical trauma during the processes of chewing, swallowing, and speaking [11,12]. Saliva contains some growth factors, such as epidermal growth factor (EGF), which improve the wound healing and soft tissue repair/regeneration[12]. It also possesses a direct antibacterial property by several protective organic components, including lysozyme, sIgA, and Lactoferrin. Specifically, lysozyme lyses the wall of bacterial cells, sIgA acts against viruses and bacteria, while Lactoferrin can inhibit the growth of bacterial cells. Moreover, saliva plays a critical role in the maintenance of pH value of the oral cavity. Bicarbonate, phosphate, and histidine-rich peptides in saliva serve as buffers to maintain the relatively neutral pH. Additionally, saliva can also influence the enamel mineralization of teeth and prevent dental caries[13].

Irradiation-induced xerostomia

Xerostomia refers to the subjective feeling of oral dryness as oppose to hyposalivation that refers to the objective saliva secretion process. Three major conditions underline salivary gland impairment including Sjogren's syndrome, an autoimmune disease, and irradiation-induced salivary impairment, and medications' xerogenic effect[14]. Each year, there are

more than 40, 000 new patients diagnosed with head and neck cancers and an estimated 8,000 deaths resulting from this disease in the United States[15]. Radiotherapy, either alone or combined with surgery and/or chemotherapy, is the most commonly used treatment for these patients. Despite the improved patients' survival rate, ionizing radiation (IR) leads to some severe side effects inevitably, due to the co-irradiation to the normal tissues surrounding the tumor. Xerostomia is one of the most prominent sequelae suffered by around 60% of patients receiving IR [16,17].

Since salivary gland cells are highly differentiated with a low mitotic activity, they should not be damaged during the acute phase of IR. However, saliva production is usually reduced shortly after IR. The exact mechanism still remains poorly understood. It has been proposed that DNA is damaged following IR due to its high radiosensitive property, leading to the reproductive cell death [18]. A study by Savage NW et al. suggested that low dose IR leads to apoptosis, while high dose leads to necrosis[19]. The reproductive cell death is the major reason for the later loss of cells, resulting in a further reduction of secretory capacity [20]. Moreover, at 24 hours post-IR, an acute inflammatory infiltrate was observed in the parenchymal cells of salivary glands, which was more common in the serous cells than the mucous cells[21]. Another proposed mechanism is that the redox-active metal ions, such as copper and iron, in the granules of cells induce the autocatalytic oxidation, leading to the lysis and disappearance of the salivary gland cells. However, both mechanisms cannot explain the fact that the reduction of saliva secretion is not consistent with the loss of cells [22]. Then, Kornings et al. proposed that plasma membrane damage induced by IR disturbs the M receptor stimulated water excretion, resulting in the reduced production of saliva in the acute phase of IR [22,23].

The compromised salivary function contributes to numerous sequelae, either directly or indirectly, and predisposes patients to morbid conditions [24]. Due to the insufficient moistening and lubrication by saliva, oral function, including speaking, chewing, and swallowing, is impeded [25]. The reduced salivary flow and insufficient lubrication also cause intolerance of denture. The patients suffer from the severe dryness of mouth and their oral mucosa is easily traumatized. The oral microflora shifts towards cariogenic bacteria, because of the reduced oral clearance and compromised buffer capacity, resulting in the rampant dental caries. Additionally, due to these changes induced by IR, the average energy intake is significantly reduced in these patients [26]. All these consequences dramatically diminish the patients' quality of life [27].

Current treatments for the irradiation-injured salivary glands

Preventive therapy

Salivary glands can be protected from IR damage by shields when the cancer is unilateral or external to the oral cavity. However, it is difficult to shield the glands without reducing the efficiency of IR, in particular for the tumors of the larynx or oral mucosa [28]. Recently, new protocols have been used in the clinic to spare the salivary glands from the IR damage, such as intensity-modulated radiation therapy with image guidance (IMRT/IGRT) [29] and proton radiotherapy [30]. IMRT is a computer-controlled high-precision radiation technique. Guided by computed tomography, the linear accelerator distributes the IR dose to the sites of cancer and/or the metastases and fit precisely to 3-D shape of the tumor by modulating the beam intensity, while avoiding co-irradiation to the surrounding healthy tissue [28]. This technique has been commonly used clinically to protect the salivary glands, especially the parotid. Although IMRT significantly reduces IR damage, ~40% of patients are still

suffering from salivary hypofunction [31]. In addition, patients with large midline cancers and bilateral metastases are unsuitable for these techniques [28]. Moreover, since the aggressive behavior of head and neck cancer, recurrence can be anticipated, additional therapeutic strategies are warranted.

Agents, such as Amifostine and Tempol, are also used to protect salivary glands against IR damage. Amifostine was developed by the U.S. Army in the 1950s to protect the soldiers from the nuclear attack. It can be dephosphorylated by alkaline phosphatase to yield an active free thiol which scavenges free radicals and reduces the IR damage [32]. It has been reported that amifostine can selectively protect the normal tissues [33], probably because the tumor vasculature has a lower alkaline phosphatase activity than the normal tissues [34]. The phase III clinical trial study demonstrated that intravenous administration of amifostine reduced the incidence of both acute and chronic xerostomia following IR, leading to the approval from FDA as an agent for protecting tissues against IR damage [35]. Interestingly, amifostine-treated patients had a lower dental caries occurrence [36]. However, the patient's 2-year survival rate was not affected by amifostine treatment [35]. Amifostine can also cause severe adverse effects, including nausea, vomiting, hypotension, and allergic reaction, especially when combined with chemotherapy, which significantly limit its application in the clinic [37]. Tempol, an alternative to amifostine, was also investigated for salivary gland protection. A study by Cotrim and her colleagues evaluated the IR protection effects of Tempol in a mouse model with IR-injured salivary glands [38]. The results demonstrated that Tempol treatment resulted in around 60% improvement on salivary flow rate without affecting the tumor growth. Moreover, the unique paramagnetic properties of Tempol allow

researchers to monitor its dynamic changes in vivo by magnetic resonance imaging (MRI). It was found that Tempol was reduced faster in tumor than in salivary glands [38].

Growth factors have been reported to possess the radioprotective potential recently. Lombaert and colleagues administered keratinocyte growth factor (KGF) pre- and/or post-irradiation to the irradiated mice. They found that KGF was able to ameliorate IR damage and restore the salivary hypofunction by re-establishing the stem/progenitor pool [39]. This growth factor is undergoing clinical trials, but unfortunately, the results of phase II trial were mixed [40]. Another study by Limesand et al. demonstrated that injection of insulin-like growth factor (IGF1) prior to the IR suppressed cell apoptosis by activating endogenous Akt of salivary acinar cells, leading to the restoration of salivary function [41]. More importantly, both IGF1 and KGF treatment had no effect on tumor growth [17,42]. In addition, it was also reported that bFGF administration prior to and immediately after IR resulted in a 44% improvement in the function of the rat parotid gland, but EGF did not show any radioprotective effect. Moreover, this study developed a sustained release system to deliver bFGF to the gland efficiently [43]. Gene transfer was also tested as an efficient delivery method for growth factors. Adenoviral vectors with bFGF or VEGF was injected through the Wharton's duct into submandibular glands of mice prior to IR. Salivary glands of mice with treatment had a significantly higher microvascular density than that of control mice [44].

Gland transplantation, especially for submandibular glands, is also an alternative strategy to spare salivary gland from the IR damage [45]. The gland could be left at the new site or returned to the original location after the radiotherapy [28]. The Seikaly-Jha method described that submandibular gland could be transferred from level 1B to the submental region under

the belly of the digastric muscle prior to the IR. After transplantation, the gland received only 5% of the total dose, leading to a reduced xerostomia occurrence [46]. However, the surgery transplantation causes some complications, such as ipsilateral facial edema, neck numbness, bleeding/hematoma formation, and so on. Moreover, it requires specialized training and more experienced surgeons.

Palliative therapies

Most of the current treatments used clinically post-IR remain palliative. These pharmacological treatments depend on the stimulation of residual acinar cells. Pilocarpine and Cevimeline, muscarinic receptor agonist, are the most commonly used drugs to stimulate saliva secretion. However, their therapeutic effects would be limited when all the functional salivary secretory cells have lost. In addition, these drugs cause many side effects, including nausea, diarrhea, and excessive sweating, which limit their application in the clinic [32]. Some artificial saliva and mouth moisturizers are also used to relieve the discomfort of patients. However, their components and properties are far away from the natural saliva. They have the lower efficiency in lubrication and cleansing due to the higher viscosity and surface tension.

Restorative therapy

Currently, three approaches are being tested to functionally restore the salivary glands damaged by IR, including gene therapy, tissue engineering, and stem-cell based therapy [15].

Gene Therapy

Salivary acinar cells which are the only site to produce fluid are extremely sensitive to ionizing irradiation. Although ductal cells are relatively radio-resistant and usually remain

intact following radiotherapy, they lack the water channels on the apical membrane. Then, a research group led by Dr. Bruce Baum proposed a gene therapy to increase water permeability in residual cells, mainly ductal cells. They developed an adenovirus encoding the human AQP1 and delivered into salivary glands via retrograde duct in a rat model [47]. The results showed that rats with ADhAQP1 had a two- to three-fold increase in salivary flow compared with the control group. However, the hAQP1 transgene was transient. The salivary flow rate of minipig dropped from 80% of baseline at 3 days post vector delivery to 69% at 7 days. Then, a non-integrating adenoviral vector, AdLTR(2)EF1α-hEPO, was introduced to extend the transgene expression for at least 2 months [48]. The effect of this gene therapy was also tested in non-human primates [49]. In this study, all adult rhesus monkeys tolerated the gene transfer without untoward local or systemic effects. However, the results were inconsistent, probably due to the inadequate perfusion of the virus into the salivary glands of primates. In addition, some other genes related to prevention of IR damage are also under investigation, such as MnSOD, glutathione peroxidase-4, and catalase. MnSOD is a mitochondrial superoxide dismutase which can defend the cells against superoxide ion. The other two enzymes can reduce the hydrogen peroxide levels [14].

Artificial salivary glands

A tissue-engineered salivary gland device was developed by Dr. Bruce Baum and Dr. Simon Tran [50]. This artificial organ is shaped like a blind-end tube with three essential components, including a biodegradable substratum, a layer of an extracellular matrix protein on the internal surface, and polarized graft cells that can secrete fluid into the lumen. Initially, several substrates and matrix proteins were compared [51]. Salivary epithelial cell line had similar behavior on poly-L-lactic acid (PLLA) and polyglycolic acid (PGA), while

PLLA gave optimal results consistently. Regarding the matrix proteins, fibronectin (FN), laminin (LN), collagen I, IV, and gelatin were tested to support the attachment and growth of graft cells. The results demonstrated that HSG cells could grow in a monolayer on the FN-coated scaffold. And LN, collagen I, and gelatin improved the growth of HSG as well. As mentioned above, the graft cells for the artificial salivary glands have to be polarized and secrete fluid unidirectionally. Due to lack of tight junctions, HSG cells are unsuitable for this artificial salivary gland device [52]. Then, a polarized salivary glands cells were successfully cultured and expanded from human submandibular glands. HuSMG cells showed appropriate polarization without any morphological or numerical abnormalities, and can be used in the artificial organ [53].

Cell-based therapy

Cell-based, especially mesenchymal stromal/stem cell (MSC)-based, therapy has been reported to hold great promise to restore the function of salivary glands injured by IR. MSCs were first introduced by AJ. Friedensten in 1974 [54]. These cells are spindle shaped and fibroblast-like. There are three minimum criteria according to the International Society of Cellular Therapy: 1) plastic culture surface adherence; 2) multilineage differentiation capacity; 3) expression of positive surface markers CD73, CD90, and CD105, while absence of the antibody markers CD45, CD34, CD14, CD11b, CD79α, CD19 and HLA-DR [55]. Currently, two types of stem cells are under investigation as experimental treatments for salivary glands repair/ regeneration, including salivary epithelial stem cells and multipotent MSCs from other tissues.

Salivary stem cell therapy

It has been well-established that the proliferating cells are mainly localized to the intercalated and excretory ducts in salivary glands [56,57]. Ligating the major duct of salivary glands resulted in the dysfunction/ apoptosis of acinar cells and proliferation of intercalated and excretory duct cells. After deligation, saliva flow is restored to normal levels. However, label-retaining cell study suggested that acinar cells had a limited proliferative activity and could not account for the restoration of salivary function [58]. Then, the group led by Robert Coppes isolated and characterized the salivary stem cells from the human excretory ducts, namely salispheres, which were able to develop into acinar-like cells within a 3D culture system [59]. Moreover, several stem cell markers of salispheres were identified, including $CD24^+$, $CD29^+$, and/or CD49f [60-62]. It was concluded that salivary stem cells not only improve the function of irradiated salivary glands, but also restore their long-term tissue homeostasis. Recently, a study from the same group showed that salispheres cultured from human salivary glands contain stem/progenitor cells which are able to self-renew and differentiate into multilineage organoids and restore the function of irradiated salivary glands in a mouse model [63]. Additionally, FACs-sorted $CD24^+$ and $CD29^+$ salisphere-derived cells from aged mice, although reduced in number, were comparable with those from young mice in terms of ex vivo expansion and post-IR regenerative potential [64]. Aaron Palmon and colleagues isolated integrin α6β1-expressing epithelial stem cells from salivary glands with a method based on magnetic affinity cell sorting (MACS) microbeads and found that these cells contain a subpopulation of salivary gland specific progenitor cells [65,66]. In addition, long-term cryopreservation did not cause the genetic or functional instability of these cells. Another study by Xiao N. et al. harvested salishpheres by isolating and culturing mouse $Lin^-CD24^+c\text{-}Kit^+Scal^+$ cells and found that glial cell line-derived

neurotrophic factor (GDNF) gene was highly expressed in these cells. Moreover, Administration of GDNF promoted salisphere growth in vitro and rescued salivary glands after irradiation without accelerating the head and neck cancer growth [67].

Multipotent MSCs from other tissues

Recently, MSCs originated from different tissues have been investigated for the restoration of irradiated salivary glands, including unselected from whole bone marrow (BM) [68], cultured from BM [69,70], cultured from adipose tissue [71,72], and from dental pulp [73]. Initially, our lab used bone marrow cells to rescue the irradiated salivary glands and reported that whole BM cells were efficient in restoring the salivary gland function [68]. Whole bone marrow-derived cells (BMDCs) were harvested from tibia and femurs of mice and transplanted through the tail vein to the mice with IR-injured SGs. The results showed that the mice with BMDCs treatment had an increased salivary output, higher cell proliferation rate and blood vessel formation, and a lower cell apoptotic activity. Moreover, stem cell markers, sca-1 and c-Kit, were higher expressed in SGs of mice with BMDCs treatment when compared with the vehicle control. Interestingly, double immunostaining confirmed that transplanted exogenous BMDCs could differentiate into epithelial acinar cells which was the major difference to other studies [74]. However, this study used unfractionated BM cells which contain a mixed population of cells, such as BM stem/ progenitor cells. It has been reported that BM stem cells could enhance the tissue repair and regeneration through differentiation and paracrine effects [75]. Then, a study by Lim used a subfractionation culturing method to isolate MSCs from whole BM cells, namely BM-cMSCs. These cells were further characterized by FACs and found to be positive for CD44, Sca-1 and negative for CD34, CD45. The results showed that injected BM-cMSCs mitigated the damage to

salivary glands following IR. In addition, transdifferentiation of MSCs was also observed in vivo. The study by Lin used a different approach. BMSCs were transdifferentiated into acinar-like cells in vitro before transplanting into irradiated mice. The results demonstrated that both BM stem cells and acinar-like cells improved the regeneration of salivary glands, but acinar-like cells showed a better therapeutic potential than those of BM stem cells[70]. All these studies reported the therapeutic effects of BM cells on hypofunction of SGs induced by IR. However, BM cell harvesting remains an invasive procedure and leads to donor discomfort [76] and in severe cases, to life-threatening complications, such as cardiopulmonary problems, bacterial infections, and cerebrovascular accidents [77].

Adipose-derived stromal cells (ADSCs), as an alternative resource of MSCs, has gained more popularity among researchers and clinicians because of its less invasive harvesting procedures and better patient compliance. Fragments of adipose tissue are minced and digested by enzymes. Then, the stromal vascular fraction (SVF) is separated with the population of mature adipocytes by centrifugation. The SVF consists of a heterogeneous population of cells, including adipose stromal, endothelial cells, hematopoietic stem/ progenitor cells, erythrocytes, fibroblasts, lymphocytes, monocyte/ macrophages, and pericytes [78,79]. When cultured, a subset of fibroblast-like cells appears and is defined as adipose-derived stromal cells (ADSCs). A study by Kojima and colleagues injected percutaneously ADSCs into submandibular glands. The results demonstrated that saliva production of ADSC-treated mice recovered to about 75% of non-irradiated controls. Moreover, ADSCs promoted vascularization and improved the tissue morphology of salivary glands. Additionally, ADSCs potentially induced the expression of various growth factors and cytokines, such as HGF, VEGF, Cox-2, and MMP-2, which might be

responsible for the therapeutic effect. ADSCs were also observed to differentiate into endothelial and ductal cells [80]. The study by Xiong et al. injected the ZsGreen-labeled human ADSCs into rat submandibular glands subcutaneously immediately following exposure to 18Gy of IR [81]. The PE-10 tubes were inserted into the Wharton's ducts to collect the stimulated saliva secreted by submandibular glands, rather than the whole saliva from the other studies. The hADSC treatment led to a higher salivary flow rate (SFR) and a greater number of acinar cells with normal morphology. Besides, ZsGreen-labeled cells co-localized with acinar (NKCC1) and ductal cells (CK7, CK14), indicating that hADSCs differentiated into acinar and ductal cells, but not myoepithelial cells. Li and colleagues administrated ADSCs by intravenous infusion into the tail vein immediately after local irradiation (18Gy). ADSCs were found to home to IR-damaged SGs within 24 hours post-injection. ADSC-treated mice had an improved saliva production, ~75% of non-IR controls, and an increased SG weight. Additionally, ADSC treatment could preserve the microscopic morphology and function of SGs with more functional acini, higher amylase production levels, enhanced cell proliferation, reduced apoptosis, and higher microvessel densities [71]. Another study by Lim and colleagues also transplanted hADSCs intravenously to restore the function of mouse SGs after 15Gy of IR. As the studies mentioned above, hADSCs injection restored SFR to ~70% of non-irradiated control mice. Some transplanted cells differentiated into amylase-positive cells. In addition, Transwell co-culture experiments showed that 13-18% of hADSCs were able to transdifferentiate into amylase-positive cells after 5 days co-culture with salivary cells which is in agreement with other studies [82].

MSCs isolated from dental tissue were also tested to rescue the IR-damaged SGs. In Yamamura and colleagues' study, dental pulp stem cells (DPSCs) were harvested from

green fluorescent protein (GFP)-expressing mice and were differentiated into dental pulp endothelial cells (DPECs) [73]. DPECs showed typical endothelial morphology and had positive expression of the gene for CD31, vascular-endothelial-cadherin, VEGF-A, and vW factor. Moreover, DPECs reorganized into a tube-like structure within Matrigel. When injected subcutaneously into nude mice, the tube-like structures representative of the injected DPECs were positive for CD31 and GFP. DPECs were also transplanted into the submandibular glands of mice with a dose of 15Gy local IR. At eight weeks post-irradiation, DPECs treatment re-established saliva production to ~60% of non-IR control mice, which was significantly higher than the vehicle-injected irradiated control mice.

Mechanisms for the therapeutic efficacy of MSC therapy

It has been well-documented that MSC-based therapy restores the hypofunction of salivary glands damaged by IR. The exact mechanism behind this therapeutic efficacy has been investigated for decades. However, it still remains poorly understood. To date, several repair mechanisms are proposed, including transdifferentiation, cell fusion, vasculogenesis, and paracrine effect [15].

Several studies mentioned above reported that transplanted MSCs, either from whole bone marrow or purified from bone marrow/ adipose tissue, could transdifferentiate into salivary epithelial cells, such as acinar and ductal cells, or endothelial cells. Thus, it was proposed that transdifferentiation may be a mechanism underlying the therapeutic effect. The transdifferentiation was also found in cell co-culture models. Maria and Tran cocultured MSCs human from bone marrow with human salivary glands cells in a co-culture system containing 2 chambers separated by a polyester membrane. The results demonstrated that up to 40% of cocultured MSCs expressed tight junction proteins and other epithelial

markers, such as aquaporin-5, α-amylase, and E-cadherin. Electron microscopy confirmed that the cellular structures of these MSCs were comparable to Human SG cells, indicating that MSCs can temporarily change into a SG acinar phenotype. Two other studies tested this cell transdifferentiation of ADSCs, originated either from mouse or human, by using similar co-culture system [72,83]. ADSCs could also adopt an epithelial phenotype when cocultured with salivary epithelial cells or conditioned medium from salivary glands. Transdifferentiation of MSCs into salivary gland cells was also reported in vivo experiments, however, only one study quantified the frequency of this observed phenomenon. Sumita and colleagues estimated approximately 9% of donor-derived salivary epithelial cells in BMDC-treated mice [68].

In vitro co-culture studies demonstrated that bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Terada and colleagues co-cultured GFP^+-bone marrow mononuclear cells isolated from female mice with male GFP^--embryonic stem cells on a gelatin-coated culture dish [84]. Within three weeks, multiple GFP^+ clones were found to be morphologically similar to embryonic stem cells. These bone marrow-derived embryonic stem-like cells (BMESL) could differentiate into various morphologies and form teratomas when injected into SCID mice. Interestingly, the genetic analyses of BMESL cells demonstrated that all BMESL clones had over-diploid DNA content, either tetraploid (4n) DNA content or hexaploid (6n) DNA content, indicating that BMESL cells were generated by cell fusion of bone marrow cells with embryonic stem cells. It is in agreement with the study by Wang X, which concluded that hepatocytes derived from bone marrow arise from cell fusion, not by differentiation of hematopoietic stem cells [85]. Another

proposed mechanism of BM-MSCs therapy was vasculogenesis from progenitors for periendothelial vascular mural and hematopoietic effector cells [86].

Although MSCs can be found to transdifferentiate into or fuse with other cells, the low frequency of cell engraftment and a low number of newly generated vascular cells could not fully explain the significant functional improvement. Moreover, some researchers have failed to detect the differentiation of MSCs in vivo study. Then, some investigators proposed an alternative mechanism that transplanted cells secrete numerous soluble factors, including growth factors, cytokines, and other paracrine factors, which are responsible for the tissue repair/ regeneration. It has been well-documented that transplanted MSCs release a broad variety of soluble factors that may be involved in cardiac repair [87]. Studies with the administration of conditioned medium (CM) from MSCs provide strong support for the paracrine paradigm. Victor J. Dzau group harvested and characterized the conditioned medium from hypoxic MSCs and injected into a rat model of permanent coronary occlusion. A dramatic reduction in infarct size and cardiac apoptosis was found after 72 hours of CM injection [88]. Another study by Takahashi observed an increased capillary density, decreased infarct size and improved cardiac function post-CM treatment [89]. Conditioned medium of adipose-derived stromal cells was also reported to have the potential to improve salivary hypofunction induced by IR. An and colleagues harvested conditioned medium from human ADSCs in hypoxic conditions (O_2< 5%) and injected into irradiated C3H mice through tail vein [90]. The conditioned secretome, containing VEGF, IL-6, IGF-1, and GM-CSF, resulted in the restoration of salivary function and increased levels of secretion of amylase and EGF. Moreover, the microscopic structural integrity of SG was preserved, and

the subpopulation of salivary glands was protected from IR damage. Interestingly, hypoxic condition promoted the anti-apoptotic effects of huADSCs secretome.

Cell extract therapy

Although transdifferentiation of MSCs was reported by using the cell tracking methods, the low frequency of cell grafting fails to explain the significant tissue repair. Recent studies suggested that MSCs contribute to tissue repair/ regeneration by releasing numerous cytokines, chemokines, and growth factors. Moreover, conditioned medium from MSCs culture had a comparable therapeutic effect with the intact cell transplantation. Based on these studies, Yeghiazarians hypothesized that if a paracrine mechanism is at play, use of the cell extract from whole bone marrow will allow us to detect the effects of any proteins or factors that would be released [91]. His team lysed the whole bone marrow cells of mice, removed the insoluble material, and kept only the soluble components after centrifugation as bone marrow cell extract. The cell extract was injected into a mouse model of myocardial infarction and resulted in the comparable therapeutic effects to the living whole BM cells, including a smaller infarct size and improved cardiac function. This approach is simple and clinically-relevant. All live cells were lysed, including stem cells, and thus, less tumorigenicity risk is associated. This extract contains numerous cytokines, chemokines, and growth factors from all types of BM cells, which could contribute to the tissue repair/ regeneration process in a well-orchestrated way. Compared to conditioned medium, BM cell extract negates the need for cell culture and thus is quicker to obtain. Moreover, BM cell extract contains less histocompatibility antigens, such as MHC-I and MHC-II, than the intact cells and theoretically has a less immunogenic effect. Yeghiazarians group also tested the therapeutic effects of cell extract originated from

human bone marrow in a mouse model of myocardial infarction. Use of cell extract does not require antibiotic therapy or the use of immunocompromised animals [92]. Additionally, our group first adopted this approach to the restoration of salivary glands damaged by irradiation [15]. Whole BM cells were lysed and soluble intracellular materials, namely 'BM Soup', were injected into the mice with irradiation-injured SGs (Figure 1) through tail vein. At 8 weeks post-irradiation, BM Soup restored salivary output, protected acinar cells, increased cell proliferation and blood vessel density, and upregulated expression of genes related to tissue repair/ regeneration (MMP2, CyclinD1, BMP7, EGF, and NGF) (Figure 2). Interestingly, both I.V. and intraglandular injections of BM Soup, and from both young and older mouse donors had comparable therapeutic effects in restoring the salivary hypofunction.

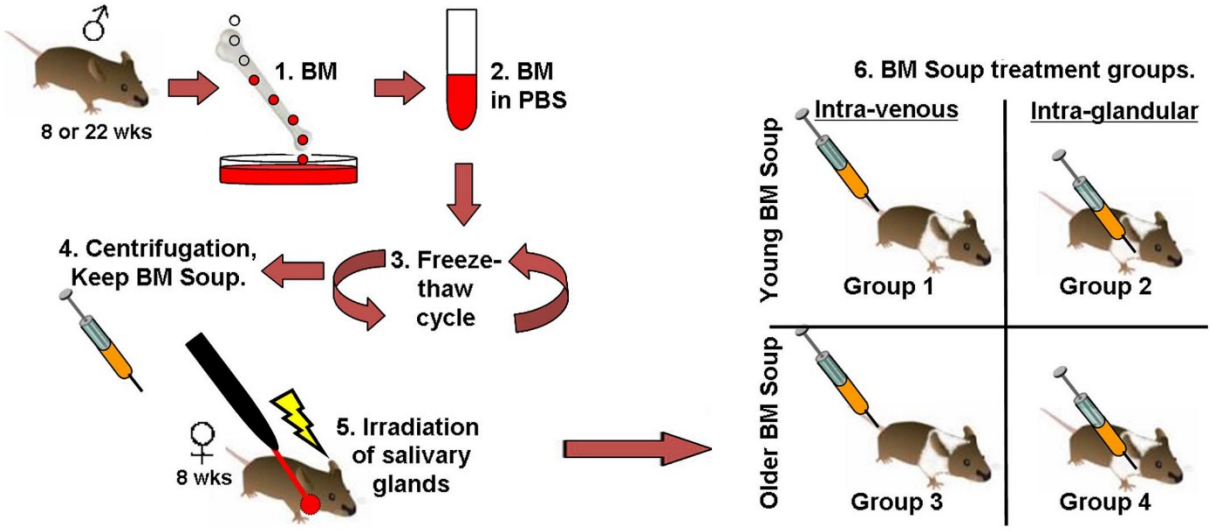

Figure 1. Bone Marrow Soup preparation and study design [15]. 1. Bone marrow is flushed from femur/tibia bones of young (8 wk-old) or older (22 wk-old) male donor mice.

2. BM cells are resuspended in PBS, strained through a filter and washed. 3. BM Soup is prepared by subjecting the BM cells to three freeze-thaw cycles of -80°C to 37°C. 4. Microcentrifugation at 17,000 g x 30 min, 4°C to remove insoluble materials. The supernatant (BM Soup) is kept on ice until injections. 5. Salivary glands of recipient female mice are irradiated with 15 Gy. 6. Within 5-7 days post-irradiation, BM Soup (from young or old donor mice) is injected intravenously or intra-glandularly. Mice were followed up for 8 weeks post-irradiation.

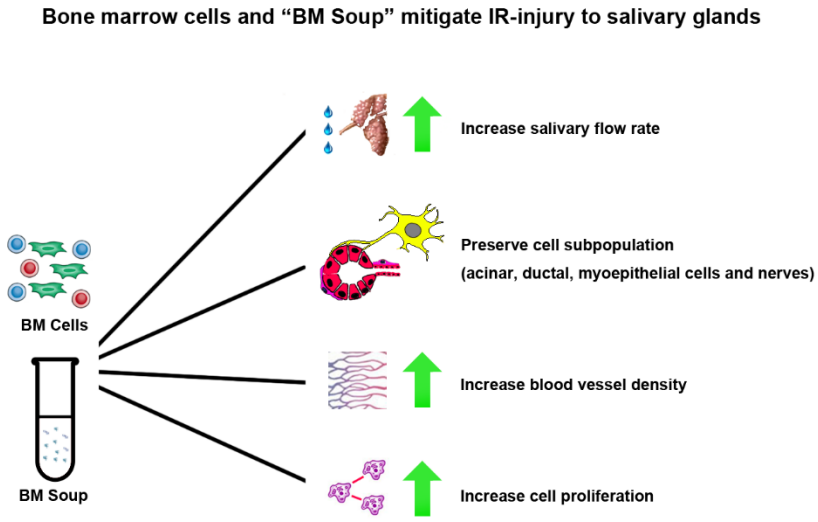

Figure 2. BM Soup mitigates the irradiation damage to salivary glands. At 8 weeks post-irradiation, BM Soup restored salivary output, protected acinar cells, increased cell proliferation and blood vessel density.

Rationale, Hypothesis, and Aims

The following three hypotheses are investigated in this thesis:

Hypothesis 1—Protein components are the bioactive ingredients in BM Soup which are responsible for the therapeutic effects.

Rationale: There are more than 40,000 new cases diagnosed with head and neck cancer annually in the United States [93]. Radiotherapy is the key component of therapy for these patients. Despite the improved patients' survival rate, irradiation causes severe side effects, among which salivary hypofunction is one of the most prominent sequelae [27]. Adult stem-cell based therapy has been reported to restore the saliva production and improve the repair/ regeneration of salivary glands. A recently proposed mechanism is that transplanted cells contribute to the tissue repair/ regeneration by releasing various cytokines, growth factor, and other paracrine factors. Our group used a cell extract from whole bone marrow cells, named BM Soup, containing numerous of these factors, to rescue the function of IR-injured salivary glands. Results showed that BM Soup restored the saliva secretion, protected salivary cells from irradiation damage, and upregulated the expression of genes related to

tissue repair/ regeneration. As a cell-free therapy, BM Soup theoretically has less immunogenicity and tumorigenicity. However, the components which are responsible for the therapeutic effects remain unknown. Identification of the active ingredients in BM Soup could enable us to detect the effects of proteins or factors released by any cell type and allow us to develop a novel molecular therapy consisted of a protein or a combination of proteins [91].

Hypothesis 2—Timing and frequency of BM Soup administration affect the therapeutic response in restoring salivary function

For any given therapy, the frequency and timing of treatment are two important parameters in the restoration of salivary glands. Our previous study only injected BM Soup between 5-7 days post-irradiation, which was considered to be during the early phase of IR damage to SGs. However, the optimal timing to initiate the BM Soup injection still remains unknown. In addition, animals were only followed for 8 weeks post-IR, and we expected that the therapeutic effect of BM Soup treatment decreases over time due to the degradation of its bioactive ingredients. Whether repeated injections of BM Soup will result in the added beneficial improvements requires further investigation. Optimizing the frequency and timing of BM Soup injections would provide the guidance for its future clinical use.

Hypothesis 3—Cell extract from different types of cells protects salivary glands from irradiation damage.

Our previous studies reported the therapeutic effects of BM Soup in the restoration of SGs. However, BM cell harvesting remains an invasive procedure and could lead to donor discomfort[76], and in severe cases, to life-threatening complications, such as

cardiopulmonary problems, bacterial infections, or cerebrovascular accidents [77]. It is necessary to test if other types of tissues, which were either clinically easier to harvest (such as the adipose tissue) or regarded as 'dispensable organ' (such as the spleen), could be used as alternate sources of "Cell Soup" for the repair of IR-injured salivary glands. These cells can be harvested with the less invasive procedure and better patient compliance.

In this thesis, a C3H mouse model of IR-injured SGs was used to test these three hypotheses. Each chapter is a manuscript published in peer-review journals or under preparation for publication.

References:

1 Tandon, A., Singh, N. N. & Sreedhar, G. Minor salivary glands and dental caries: Approach towards a new horizon. *J Nat Sci Biol Med* **4**, 364-368 (2013).

2 Perez, P., Rowzee, A. M., Zheng, C., Adriaansen, J. & Baum, B. J. Salivary epithelial cells: an unassuming target site for gene therapeutics. *Int J Biochem Cell Biol* **42**, 773-777 (2010).

3 F. Christopher Holsinger, D. T. B. in *Salivary Gland Disorders* (ed Robert L. Ferris Eugene N. Myers) Ch. 1, 1-16 (Springer Berlin Heidelberg, 2007).

4 Catalan, M. A., Nakamoto, T. & Melvin, J. E. The salivary gland fluid secretion mechanism. *J Med Invest* **56 Suppl**, 192-196 (2009).

5 Emmelin, N. & Gjorstrup, P. On the function of myoepithelial cells in salivary glands. *J Physiol* **230**, 185-198 (1973).

6 Cook DI, Y. J. in *Handbook of Physiology, The Gastrointestinal System, Salivary, Gastric, Pancreatic, and Hepatobiliary Secretion* Ch. 1-23, (Wiley-blackwell, 1989).

7 Turner, R. J. & Sugiya, H. Understanding salivary fluid and protein secretion. *Oral Dis* **8**, 3-11 (2002).

8 Proctor, G. B. & Carpenter, G. H. Regulation of salivary gland function by autonomic nerves. *Auton Neurosci* **133**, 3-18 (2007).

9 Culp, D. J., Luo, W., Richardson, L. A., Watson, G. E. & Latchney, L. R. Both M1 and M3 receptors regulate exocrine secretion by mucous acini. *Am J Physiol* **271**, C1963-1972 (1996).

10 R.B., B. B. J. W. in *Neural Mechanisms of Salivary Gland Secretion* Vol. 11 (ed Ekström J Garrett JR, Anderson LC) Ch. 3, 44-58 (Basel, Karger, 1999).

11 Tabak, L. A., Levine, M. J., Mandel, I. D. & Ellison, S. A. Role of salivary mucins in the protection of the oral cavity. *J Oral Pathol* **11**, 1-17 (1982).

12 Mandel, I. D. The functions of saliva. *J Dent Res* **66 Spec No**, 623-627 (1987).

13 Shaw, J. H. & Wollman, D. H. The influence of sialoadenectomy in rats on food and water consumption. *J Dent Res* **37**, 805-810 (1958).

14 Vitolo, J. M. & Baum, B. J. The use of gene transfer for the protection and repair of salivary glands. *Oral Dis* **8**, 183-191 (2002).

15 Tran, S. D. *et al.* Paracrine effects of bone marrow soup restore organ function, regeneration, and repair in salivary glands damaged by irradiation. *PLoS One* **8**, e61632 (2013).
16 Vissink, A. *et al.* Clinical management of salivary gland hypofunction and xerostomia in head-and-neck cancer patients: successes and barriers. *Int J Radiat Oncol Biol Phys* **78**, 983-991 (2010).
17 Zheng, C. *et al.* Prevention of radiation-induced salivary hypofunction following hKGF gene delivery to murine submandibular glands. *Clin Cancer Res* **17**, 2842-2851 (2011).
18 Nagler, R. M. The enigmatic mechanism of irradiation-induced damage to the major salivary glands. *Oral Dis* **8**, 141-146 (2002).
19 Savage, N. W., Kruger, B. J. & Adkins, K. F. The effects of fractionated megavoltage X-irradiation on rat submandibular gland: an assessment by electron microscopy. *Aust Dent J* **30**, 188-193 (1985).
20 Zeilstra, L. J., Vissink, A., Konings, A. W. & Coppes, R. P. Radiation induced cell loss in rat submandibular gland and its relation to gland function. *Int J Radiat Biol* **76**, 419-429 (2000).
21 Kashima HK, K. W., Andrews JR. Postirradiation Sialadenitis – a Study of Clinical Features Histopathologic Changes and Serum Enzyme Variations Following Irradiation of Human Salivary Glands. *American Journal of Roentgenology Radium Therapy and Nuclear Medicine* **94**, 271-179 (1965).
22 Konings, A. W., Coppes, R. P. & Vissink, A. On the mechanism of salivary gland radiosensitivity. *Int J Radiat Oncol Biol Phys* **62**, 1187-1194 (2005).
23 Coppes, R. P., Vissink, A. & Konings, A. W. Comparison of radiosensitivity of rat parotid and submandibular glands after different radiation schedules. *Radiother Oncol* **63**, 321-328 (2002).
24 Vissink, A., Jansma, J., Spijkervet, F. K., Burlage, F. R. & Coppes, R. P. Oral sequelae of head and neck radiotherapy. *Crit Rev Oral Biol Med* **14**, 199-212 (2003).
25 Hamlet, S. *et al.* Mastication and swallowing in patients with postirradiation xerostomia. *Int J Radiat Oncol Biol Phys* **37**, 789-796 (1997).
26 Backstrom, I., Funegard, U., Andersson, I., Franzen, L. & Johansson, I. Dietary intake in head and neck irradiated patients with permanent dry mouth symptoms. *Eur J Cancer B Oral Oncol* **31b**, 253-257 (1995).
27 Fang, D. *et al.* Identification of the active components in Bone Marrow Soup: a mitigator against irradiation-injury to salivary glands. *Sci Rep* **5**, 16017 (2015).
28 Redman, R. S. On approaches to the functional restoration of salivary glands damaged by radiation therapy for head and neck cancer, with a review of related aspects of salivary gland morphology and development. *Biotech Histochem* **83**, 103-130 (2008).
29 Trotti, A. & Eisbruch, A. Reducing xerostomia through advanced technology. *Lancet Oncol* **12**, 110-111 (2011).
30 Holliday, E. B. & Frank, S. J. Proton radiation therapy for head and neck cancer: a review of the clinical experience to date. *Int J Radiat Oncol Biol Phys* **89**, 292-302 (2014).
31 Vergeer, M. R. *et al.* Intensity-modulated radiotherapy reduces radiation-induced morbidity and improves health-related quality of life: results of a nonrandomized prospective study using a standardized follow-up program. *Int J Radiat Oncol Biol Phys* **74**, 1-8 (2009).
32 Grundmann, O., Mitchell, G. C. & Limesand, K. H. Sensitivity of salivary glands to radiation: from animal models to therapies. *J Dent Res* **88**, 894-903 (2009).
33 Utley, J. F., Marlowe, C. & Waddell, W. J. Distribution of 35S-labeled WR-2721 in normal and malignant tissues of the mouse1,2. *Radiat Res* **68**, 284-291 (1976).
34 Calabro-Jones, P. M., Fahey, R. C., Smoluk, G. D. & Ward, J. F. Alkaline phosphatase promotes radioprotection and accumulation of WR-1065 in V79-171 cells incubated in

medium containing WR-2721. *Int J Radiat Biol Relat Stud Phys Chem Med* **47**, 23-27 (1985).
35 Brizel, D. M. *et al.* Phase III randomized trial of amifostine as a radioprotector in head and neck cancer. *J Clin Oncol* **18**, 3339-3345 (2000).
36 Rudat, V. *et al.* Protective effect of amifostine on dental health after radiotherapy of the head and neck. *Int J Radiat Oncol Biol Phys* **48**, 1339-1343 (2000).
37 Rades, D. *et al.* Serious adverse effects of amifostine during radiotherapy in head and neck cancer patients. *Radiother Oncol* **70**, 261-264 (2004).
38 Cotrim, A. P. *et al.* Differential radiation protection of salivary glands versus tumor by Tempol with accompanying tissue assessment of Tempol by magnetic resonance imaging. *Clin Cancer Res* **13**, 4928-4933 (2007).
39 Lombaert, I. M. *et al.* Keratinocyte growth factor prevents radiation damage to salivary glands by expansion of the stem/progenitor pool. *Stem Cells* **26**, 2595-2601 (2008).
40 Brizel, D. M. *et al.* Phase II study of palifermin and concurrent chemoradiation in head and neck squamous cell carcinoma. *J Clin Oncol* **26**, 2489-2496 (2008).
41 Limesand, K. H., Said, S. & Anderson, S. M. Suppression of radiation-induced salivary gland dysfunction by IGF-1. *PLoS One* **4**, e4663 (2009).
42 Victory, K. *et al.* Head and neck tumor cell radiation response occurs in the presence of IGF1. *J Dent Res* **90**, 347-352 (2011).
43 Thula, T. T., Schultz, G., Tran-Son-Tay, R. & Batich, C. Effects of EGF and bFGF on irradiated parotid glands. *Ann Biomed Eng* **33**, 685-695 (2005).
44 Cotrim, A. P., Sowers, A., Mitchell, J. B. & Baum, B. J. Prevention of irradiation-induced salivary hypofunction by microvessel protection in mouse salivary glands. *Mol Ther* **15**, 2101-2106 (2007).
45 Sood, A. J. *et al.* Salivary gland transfer to prevent radiation-induced xerostomia: a systematic review and meta-analysis. *Oral Oncol* **50**, 77-83 (2014).
46 Seikaly, H. *et al.* Submandibular gland transfer: a new method of preventing radiation-induced xerostomia. *Laryngoscope* **111**, 347-352 (2001).
47 Delporte, C. *et al.* Increased fluid secretion after adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands. *Proc Natl Acad Sci U S A* **94**, 3268-3273 (1997).
48 Zheng, C. *et al.* Extended transgene expression from a nonintegrating adenoviral vector containing retroviral elements. *Mol Ther* **16**, 1089-1097 (2008).
49 O'Connell, A. C. *et al.* Safety and efficacy of adenovirus-mediated transfer of the human aquaporin-1 cDNA to irradiated parotid glands of non-human primates. *Cancer Gene Ther* **6**, 505-513 (1999).
50 Baum, B. J. & Tran, S. D. Synergy between genetic and tissue engineering: creating an artificial salivary gland. *Periodontol 2000* **41**, 218-223 (2006).
51 Aframian, D. J. *et al.* The growth and morphological behavior of salivary epithelial cells on matrix protein-coated biodegradable substrata. *Tissue Eng* **6**, 209-216 (2000).
52 Aframian, D. J., Tran, S. D., Cukierman, E., Yamada, K. M. & Baum, B. J. Absence of tight junction formation in an allogeneic graft cell line used for developing an engineered artificial salivary gland. *Tissue Eng* **8**, 871-878 (2002).
53 Tran, S. D. *et al.* Primary culture of polarized human salivary epithelial cells for use in developing an artificial salivary gland. *Tissue Eng* **11**, 172-181 (2005).
54 Friedenstein, A. J., Chailakhyan, R. K., Latsinik, N. V., Panasyuk, A. F. & Keiliss-Borok, I. V. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation* **17**, 331-340 (1974).

55 Dominici, M. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315-317 (2006).
56 Denny, P. C., Chai, Y., Klauser, D. K. & Denny, P. A. Parenchymal cell proliferation and mechanisms for maintenance of granular duct and acinar cell populations in adult male mouse submandibular gland. *Anat Rec* **235**, 475-485 (1993).
57 Man, Y. G., Ball, W. D., Marchetti, L. & Hand, A. R. Contributions of intercalated duct cells to the normal parenchyma of submandibular glands of adult rats. *Anat Rec* **263**, 202-214 (2001).
58 Pringle, S., Van Os, R. & Coppes, R. P. Concise review: Adult salivary gland stem cells and a potential therapy for xerostomia. *Stem Cells* **31**, 613-619 (2013).
59 Lombaert, I. M. *et al.* Rescue of salivary gland function after stem cell transplantation in irradiated glands. *PLoS One* **3**, e2063 (2008).
60 Feng, J., van der Zwaag, M., Stokman, M. A., van Os, R. & Coppes, R. P. Isolation and characterization of human salivary gland cells for stem cell transplantation to reduce radiation-induced hyposalivation. *Radiother Oncol* **92**, 466-471 (2009).
61 Coppes, R. P. & Stokman, M. A. Stem cells and the repair of radiation-induced salivary gland damage. *Oral Dis* **17**, 143-153 (2011).
62 Nanduri, L. S. *et al.* Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. *Radiother Oncol* **108**, 458-463 (2013).
63 Pringle, S. *et al.* Human Salivary Gland Stem Cells Functionally Restore Radiation Damaged Salivary Glands. *Stem Cells* **34**, 640-652 (2016).
64 Maimets, M., Bron, R., de Haan, G., van Os, R. & Coppes, R. P. Similar ex vivo expansion and post-irradiation regenerative potential of juvenile and aged salivary gland stem cells. *Radiother Oncol* **116**, 443-448 (2015).
65 Palmon, A. *et al.* High-efficiency immunomagnetic isolation of solid tissue-originated integrin-expressing adult stem cells. *Methods* **56**, 305-309 (2012).
66 Neumann, Y. *et al.* Long-term cryopreservation model of rat salivary gland stem cells for future therapy in irradiated head and neck cancer patients. *Tissue Eng Part C Methods* **18**, 710-718 (2012).
67 Xiao, N. *et al.* Neurotrophic factor GDNF promotes survival of salivary stem cells. *J Clin Invest* **124**, 3364-3377 (2014).
68 Sumita, Y. *et al.* Bone marrow-derived cells rescue salivary gland function in mice with head and neck irradiation. *Int J Biochem Cell Biol* **43**, 80-87 (2011).
69 Lim, J. Y. *et al.* Intraglandular transplantation of bone marrow-derived clonal mesenchymal stem cells for amelioration of post-irradiation salivary gland damage. *Oral Oncol* **49**, 136-143 (2013).
70 Lin, C. Y. *et al.* Cell therapy for salivary gland regeneration. *J Dent Res* **90**, 341-346 (2011).
71 Li, Z. *et al.* Protective efficacy of intravenous transplantation of adipose-derived stem cells for the prevention of radiation-induced salivary gland damage. *Arch Oral Biol* **60**, 1488-1496 (2015).
72 Lim, J. Y. *et al.* Systemic transplantation of human adipose tissue-derived mesenchymal stem cells for the regeneration of irradiation-induced salivary gland damage. *PLoS One* **8**, e71167 (2013).
73 Yamamura, Y. *et al.* Treatment of salivary gland hypofunction by transplantation with dental pulp cells. *Arch Oral Biol* **58**, 935-942 (2013).
74 Lombaert, I. M. *et al.* Mobilization of bone marrow stem cells by granulocyte colony-stimulating factor ameliorates radiation-induced damage to salivary glands. *Clin Cancer Res* **12**, 1804-1812 (2006).

75 Wu, Y., Chen, L., Scott, P. G. & Tredget, E. E. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* **25**, 2648-2659 (2007).
76 Stroncek, D. F. *et al.* Experiences of the first 493 unrelated marrow donors in the National Marrow Donor Program. *Blood* **81**, 1940-1946 (1993).
77 Buckner, C. D. *et al.* Marrow harvesting from normal donors. *Blood* **64**, 630-634 (1984).
78 Bunnell, B. A., Flaat, M., Gagliardi, C., Patel, B. & Ripoll, C. Adipose-derived stem cells: isolation, expansion and differentiation. *Methods* **45**, 115-120 (2008).
79 Bourin, P. *et al.* Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* **15**, 641-648 (2013).
80 Kojima, T. *et al.* Regeneration of radiation damaged salivary glands with adipose-derived stromal cells. *Laryngoscope* **121**, 1864-1869 (2011).
81 Xiong, X., Shi, X. & Chen, F. Human adipose tissue-derived stem cells alleviate radiation-induced xerostomia. *International Journal of Molecular Medicine* **34**, 749-755 (2014).
82 Maria, O. M. & Tran, S. D. Human mesenchymal stem cells cultured with salivary gland biopsies adopt an epithelial phenotype. *Stem Cells Dev* **20**, 959-967 (2011).
83 Lee, J., Park, S. & Roh, S. Transdifferentiation of mouse adipose-derived stromal cells into acinar cells of the submandibular gland using a co-culture system. *Exp Cell Res* **334**, 160-172 (2015).
84 Terada, N. *et al.* Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* **416**, 542-545 (2002).
85 Wang, X. *et al.* Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* **422**, 897-901 (2003).
86 Rajantie, I. *et al.* Adult bone marrow–derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. *Blood* **104**, 2084-2086 (2004).
87 Caplan, A. I. & Dennis, J. E. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* **98**, 1076-1084 (2006).
88 Gnecchi, M. *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* **11**, 367-368 (2005).
89 Takahashi, M. *et al.* Cytokines produced by bone marrow cells can contribute to functional improvement of the infarcted heart by protecting cardiomyocytes from ischemic injury. *Am J Physiol Heart Circ Physiol* **291**, H886-893 (2006).
90 An, H.-Y. *et al.* Adipose Mesenchymal Stem Cell Secretome Modulated in Hypoxia for Remodeling of Radiation-Induced Salivary Gland Damage. *PLoS ONE* **10**, e0141862 (2015).
91 Yeghiazarians, Y. *et al.* Injection of bone marrow cell extract into infarcted hearts results in functional improvement comparable to intact cell therapy. *Mol Ther* **17**, 1250-1256 (2009).
92 Angeli, F. S. *et al.* Injection of human bone marrow and mononuclear cell extract into infarcted mouse hearts results in functional improvement. *Open Cardiovasc Med J* **6**, 38-43 (2012).
93 Siegel, R., Ma, J., Zou, Z. & Jemal, A. Cancer statistics, 2014. *CA Cancer J Clin* **64**, 9-29 (2014).

Chapter 2-Identification of the active components in Bone Marrow Soup: a mitigator against irradiation-injury to salivary glands.

Preface

Our previous study used a cell extract from whole bone marrow cells, named BM Soup, which contains numerous cytokines, growth factor, and other paracrine factors, to rescue the function of IR-injured SGs. The results demonstrated that BM Soup restored the saliva secretion, protected salivary cells from IR damage, and upregulated the expression of genes for tissue repair/ regeneration. However, the components which are responsible for the therapeutic effects remain unknown.

In this chapter, we identified that the native proteins (but not the nucleic acids, lipids or carbohydrates) were the therapeutic ingredients in BM Soup for functional salivary restoration following IR. In addition, Protein arrays were used to detect several angiogenesis-related factors (CD26, FGF, HGF, MMP-8, MMP-9, OPN, PF4, and SDF-1) and cytokines (IL-1ra, IL-16) in BM Soup.

The study presented in this chapter has been published in the journal of Scientific Reports, 5, 16017; doi: 10.1038/srep16017 (2015) © Nature Publishing Group.

Identification of the active components in Bone Marrow Soup: a mitigator against irradiation-injury to salivary glands.

Running title: Ingredients in mitigator against radiation-injury

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Abstract

In separate studies, an extract of soluble intracellular contents from whole bone marrow cells, named "Bone Marrow (BM) Soup", was reported to either improve cardiac or salivary functions post-myocardial infarction or irradiation (IR), respectively. However, the active components in BM Soup are unknown. To demonstrate that proteins were the active ingredients, we devised a method using proteinase K followed by heating to deactivate proteins and for safe injections into mice. BM Soup and "deactivated BM Soup" were injected into mice that had their salivary glands injured with 15Gy IR. Control mice received either injections of saline or were not IR. Results at week 8 post-IR showed the 'deactivated BM Soup' was no better than injections of saline, while injections of native BM Soup restored saliva flow, protected salivary cells and blood vessels from IR-damage. Protein arrays detected several angiogenesis-related factors (CD26, FGF, HGF, MMP-8, MMP-9, OPN, PF4, SDF-1) and cytokines (IL-1ra, IL-16) in BM Soup. In conclusion, the native proteins (but not the nucleic acids, lipids or carbohydrates) were the therapeutic ingredients in BM Soup for functional salivary restoration following IR. This molecular therapy approach has clinical potential because it is theoretically less tumorigenic and immunogenic than cell therapies.

Key words: Molecular therapy, Radiation injury, Salivary gland, Cell therapy, Cell extract, Head and neck cancer, Bone marrow, Growth factors, Cytokines

Introduction

During the past decade, the number of patients newly diagnosed with head and neck cancer has doubled to more than 40,000 in the United States[1, 2]. Surgery and irradiation (IR) remain the most frequently used treatments for head and neck cancer. Despite an improved patients' survival rate, IR causes inevitably severe side effects, due to the high dose of co-irradiation to normal tissue surrounding the tumor, among which salivary hypofunction (dry mouth, xerostomia) is the most prominent sequela experienced by more than 60% of patients receiving IR for head and neck cancer[3, 4]. To reduce IR damage to the salivary glands (SG), new radiation techniques such as intensity-modulated radiation therapy with image guidance (IMRT/IGRT) and proton radiotherapy are being used[5, 6]. Submandibular gland transfer is also an alternative method to spare SG from high dose co-irradiation[7]. However, ~40% of patients receiving IMRT are still suffering from reduced salivary flow[8], and gland transfer cannot be applied to every patient. Salivary hypofunction (i.e., reduced saliva flow) predisposes patients to morbid conditions such as oral mucositis and infections, dental caries, difficultly speaking, chewing and swallowing food and leading to a diminished quality of life and malnutrition[9]. Unfortunately, current treatments for salivary hypofunction remain palliative and thus radical treatment strategies are required to restore SG function. Gene therapy, tissue engineering and stem cells are currently the three major experimental approaches tested for functional restoration of damaged SG.

Adult stem cell-based therapy has been reported to slow down the apoptotic activity[10], to normalize the stem/ progenitor cell pool[9], and to improve the function of SG. Several types of stem cells, such as bone marrow (BM)[10, 11], adipose-derived stromal cells[12], dental pulp cells[13] and SG stem / progenitor cells[9], were reported to restore function of SG damaged

by IR. Initially, the mechanisms of (therapeutic) action proposed that stem cells differentiated into or fused with the tissue parenchymal cells[14, 15]. The currently proposed mechanism is that transplanted cells (e.g., BM or MSC) secrete paracrine factors, such as cytokines and growth factors, needed for the tissue repair and regeneration process[16, 17]. Yeghiazarians and colleagues elegantly demonstrated that injection of intact BM cells versus a cell lysate (bone marrow cell extract) resulted in comparable benefits in a mouse model of acute myocardial infarction[18]. However when the whole BM was fractionated into subpopulations of cells ('fractionated BM cell extract'), then the cardiac functional efficacy was less than that of 'whole BM cell extract'[19]. These results suggested that numerous factors secreted/ released by numerous BM cell populations were responsible for cardiac functional improvement. Interestingly, the same group of researchers injected 'human' BM cell extract into the same mouse model and reported improved cardiac function without immune rejection[20]. Thus this BM cell extract is less immunogenic than transplanting whole BM cells. Our group adapted this method and coined the term as "Bone Marrow Soup" *(BM Soup*), which represented all the yet-to-be-identified soluble components of the cell lysate from whole bone marrow cells. We reported that both whole BM cells and BM Soup restored comparable salivary function following IR and in Sjogren's-like disease mice[21, 22]. Because the BM Soup is an extract from a cell lysate, it is theoretically less immunogenic and tumorigenic[23]. However, the components which are responsible for these promising therapeutic actions remain unknown.

In this study, we have demonstrated that the protein components but not the nucleic acids, lipids, or carbohydrates in the BM Soup are the active/therapeutic ingredients for functional salivary restoration following IR. We have also employed a protein microarray approach

to preliminarily identify important cytokines and growth factors that are present in the BM Soup.

Results

Proteins are the active ingredients in BM Soup

Four methods to deactivate proteins were tested in this study: 1) 95°C for 20 min, 2) 95°C for 1hour, 3) trypsin digestion at 37°C for overnight, and 4) proteinase K at 37°C for overnight. Coomassie blue staining was used to visualize the proteins after deactivation **(Fig. 1a-b)**. All four methods cleaved/degraded the majority of proteins above 12 kDa. Coomassie staining indicated that the total density for remaining proteins after deactivation from all four methods was 80~90% less intense than that of the native BM Soup **(Fig. 1c)**. Measurements of protein concentration (BCA method) showed a reduction ~20% with trypsin and ~50% with heat or proteinase K alone **(Fig. 1d)**. The proteinase K method resulted in the least amount of proteins observed on the gel and with the smallest peptides. Thus we chose proteinase K (0.1 μg/μl) as the most efficient method to degrade/deactivate proteins from BM Soup. However, considering the toxicity of proteinase K if injected into live animals, an additional step of heating at 95°C for 20 min was conducted to inactivate proteinase K **(Fig. 1b)**. Almost no protein bands were observed on the gel with Commassie blue staining.

In vivo injection of 'Deactivated BM Soup' does not restore function in irradiated salivary glands to normal levels.

Salivary flow rate (SFR) is an objective measure of SG function. Results at week 8 post-IR showed that SFR of irradiated mice injected with the vehicle control (saline control;

IR+SC group) had a significantly reduced SFR ($P<0.05$, n=5) when compared with sham-IR mice (i.e. the negative control group; mice were sedated and placed inside the irradiator but the machine was off; **Fig. 2a**). SFR of irradiated mice injected with the 'Deactivated BM Soup' was comparable to that of irradiated mice injected with saline control (IR+SC group). This meant that the 'Deactivated BM Soup' was no better than injections of normal saline in IR mice. SFR of mice in the BM Soup-treated group was comparable to that of sham-IR mice. These data indicated that BM Soup treatment, not the deactivated BM Soup, restored saliva secretory function (**Fig. 2a**, $P<0.05$, n=5). Because the 'Deactivated BM Soup' had its active proteins cleaved by proteinase K (while nucleic acids, lipids and carbohydrates were not), these results suggested that the proteins were potential therapeutic ingredients in BM Soup.

In addition to monitoring SFR, we also compared the 'native BM Soup' and its deactivated form at the histology, gene, and protein expression levels in irradiated SG. All these measures confirmed the same trend observed with SFR. That is, 'deactivated BM Soup' was not better than injection of saline while the native BM Soup protected salivary cells and blood vessels from IR damage, and up-regulated the expression of genes related to SG development and regeneration. Thus the loss of therapeutic effect of the BM Soup was due to the removal (deactivation) of its native protein components.

The percentage of surface acinar area/ total area was comparable between sham-IR and BM soup-treated groups, 70.6% ± 3.2% and 71.6% ± 5.5% respectively, which were significantly higher than the deactivated BM Soup and saline control groups ($P<0.05$, **Fig. 2b**). Histological observations showed that the acini structure was damaged in the saline control group and the deactivated BM Soup group, but well preserved in BM Soup group

(**Fig. 2c-f**). Von Willebrand Factor staining showed that the number of blood vessels in saline control or deactivated BM soup-treated group was ~50% lowered than blood vessels in the sham-IR and BM soup-treated mice ($P<0.05$, **Fig. 2g**). PCNA staining revealed that BM soup-treated mice had a higher percentage of proliferating cells than the deactivated BM soup group (**Fig. 2h**, $P<0.05$). Subpopulations of salivary cells were detected by immunofluorescent staining. More cells were positive for Aquaporin 5 (AQP5, marker of acinar cells), alpha-smooth muscle actin (α-SMA, marker for myoepithelial cells), Keratin 5 (CK5) and c-Kit (markers for ductal and progenitor cells), and GDNF family receptor alpha-2 (GFRα2, marker for parasympathetic nerves) were observed in BM Soup-treated group when compared with the IR+SC or deactivated BM Soup treatment (**Fig. 3**, $P<0.05$). The expression levels of genes related to tissue repair/regeneration were analyzed by quantitative RT-PCR **(Fig. 4)**. All genes tested in this study were up-regulated after BM Soup treatment. Specifically, expression of AQP5 (acinar cell), CK5 (ductal cell), VEGF, NGF and Cyclin D1 (cell cycle G1/Synthesis) were significantly higher in BM soup-treated group than that of the deactivated BM soup ($P<0.05$).

BM Soup contained angiogenesis growth factors and cytokines that may play important role in functional SG restoration

We hypothesized that functional restoration of SGs is attributed to the presence of protein factors secreted/released by bone marrow cells. Thus a protein microarray approach was used to characterize the angiogenesis-related growth factors in BM Soup. Several angiogenesis-related proteins were identified in BM soup **(Fig. 5a)**. Specifically, factors related to tissue repair/ regeneration such as MMP-8, -9, FGF-1, HGF, OPN and SDF-1,

and some anti-angiogenic factors (PF4 and CD26) were found to be present in BM Soup. A cytokine microarray was also used to profile cytokines in the BM Soup. Two cytokines, IL-1ra and IL-16, were found to be at abundant levels in BM Soup **(Fig. 5e)**. After the deactivation process using proteinase K + 95°C, all these factors and cytokines were below the detection level of the two protein arrays used in this study **(Fig. 5b, f)**. A negative control group using normal saline injection into IR mice was also tested on the protein arrays **(Fig. 5c, g)**. Overall, the protein array results demonstrated that: 1) Proteinase K and heat were efficient in decreasing the proteins that were initially detected on the array for the 'Native BM Soup' **(Fig 5a, 5e)**, and 2) a preliminarily screen of some angiogenesis-related growth factors and cytokines in the 'Native BM Soup' was done but we have not confirmed yet which of these factors were responsible for the therapeutic effects. The limitations of protein arrays are enumerated in the Discussion section.

Discussion

The findings of this study were: 1) that the protein components in the BM Soup were the active/ therapeutic ingredients for functional salivary restoration following IR, and 2) that some of the protein components in BM Soup were angiogenesis-related growth factors and cytokines.

To confirm protein factors were the active ingredients, BM Soup was digested by proteinase K ("Deactivated BM Soup") and injected into head and neck IR mice. The strategy of using proteinase K combined with heating at 95°C served three purposes. First, this method allowed the injection of the "Deactivated BM Soup" safely into mice. Second, proteinase K cleaved active proteins into (smaller) peptides/ amino acids and this would suggest that the active proteins were the therapeutic ingredients in BM Soup (and not the

cleaved peptides). Third, proteinase K is commonly used in molecular biology to digest proteins and remove contamination from preparations of nucleic acid (i.e., to isolate highly native, undamaged DNAs or RNAs). Thus for nucleic acids, the BM Soup that was treated with proteinase K would still have native (or active) nucleic acids. Our hypothesis behind this study is that proteins (rather than DNA, lipid or carbohydrate) are the main molecules that execute the cellular function (intracellularly or extracellularly). While lipid and carbohydrate are stable molecules that would survive the proteinase K with heat treatment, we know that heating at 95 degrees denatures double stranded DNA. However, we do not think DNA plays an important role of stimulating development, regeneration and differentiation of the salivary gland cells. For lipids and carbohydrates, there are no reports in the literature regarding the cleavage of lipids and carbohydrates by proteinase K. Also based on our experience with biochemistry, lipids and carbohydrates should be stable at a temperature of 95°C. There are two protease inhibitors that can totally inactivate proteinase K, which are Diisopropyl fluorophosphates (DFP) and phenylmethylsulfonyl fluoride (PMSF). DFP is much more toxic than PMSF. PMSF covalently binds to the serine amino acid present in the active site of the protease. Any remaining PMSF is rapidly degraded in water. The half-life in aqueous solutions is 110 min at pH 7, 55 min at pH 7.5, and 35 min at pH 8, all at 25°C. However, we were concerned that both proteinase K and PMSF would still be toxic when injected into IR mice and thus demonstrated that heating with 95°C removed the 28.9 kDa band (proteinase K) from the gel **(Fig. 1b)**.

Although our chosen method of proteinase K with 95°C heating seems to be simple minded, it made the point, based on our experience in bioanalytical chemistry, that proteins (and not DNA, RNA, lipids, carbohydrates) were the active ingredients in BM Soup that are

responsible for the treatment. There are of course other strategies, based on our experience in bioanalytical chemistry and from recommendations through the review of this study, which could have been used. For example, if the purpose was to rule out the role of small molecules (such as lipids or carbohydrates) in the BM Soup treatment, then using ultracentrifugation and a 3 kDa cutoff filter (such as the Amicon Ultra Filter) would have contained the proteins, nucleic acids, and other large molecules (everything above 3 kDa) in the filter top while the filtrate (flow-through) would contain lipids, carbohydrates, amino acids, and small peptides. Injecting the components in the filter top would have resulted in a positive effect in irradiated SG while injections of the filtrate into IR mice would not have any treatment effect. Alternatively, we could use an approach consisting of "BM Soup + proteinase K + Amicon Ultra Filter (3K Cutoff)". The filter top would only contain proteinase K (and some large peptides derived from the proteinase K cleavage) while the filtrate would contain lipids, carbohydrates, amino acids, peptides (including those derived from protein K-cleaved proteins). Both filter top and filtrate should not have any treatment effect if injected into IR mice. Additional methods to separate specific components in BM Soup could have been protein deglycosylation, isolation of lipid droplets from cells by density gradient centrifugation, or treatment with nucleases to remove nucleic acids. However with every additional method of separation, there would be a requirement for a higher number of IR mice for the *in vivo* injection experiments. Thus we selected, based on our experience with biochemistry, the use of proteinase K plus 95°C heating as the most convenient and straightforward method to demonstrate proteins were the active ingredients in BM Soup.

Cotrim and coworkers tested the notion that injury to the adjacent microvasculature played a role in SG radiation damage by demonstrating that FGF and VEGF gene transfer protected mouse SG endothelial cells that underwent head and neck IR[24]. Results from our study also indicated a significant reduction in the number of micro-vessels in irradiated SG. Thus, we focused on identifying angiogenesis-related factors in BM Soup using a protein microarray approach. The microarray analysis indicated that matrix metallo-proteinase-8 (MMP-8), MMP-9 and osteopontin (OPN) were among the highly expressed factors in BM Soup. Their roles in angiogenesis were reported as such: MMP-8 and -9 degraded the matrix surrounding endothelial cells, and then promoted their migration and proliferation, resulting in angiogenesis[25, 26]. OPN inhibited the apoptosis of vascular endothelial cells, promoted their migration, and induced angiogenesis of endothelial progenitor cells[27, 28]. FGF-1 and HGF, another two growth factors expressed in BM soup, were reported to participate in the development of SG[29, 30]. Stromal cell derived factor-1 (SDF-1) and its receptor CXCR4 play a critical role in the migration of cells and other biological process, including vascularization, immune response and neurogenesis[31, 32, 33, 34]. SDF-1 expression is increased during the response to tissue injury and induces progenitor/ stem cells with CXCR4 to migrate from the bone marrow to the damage site[35]. Wang and colleagues reported that the SDF-1/CXCR4 pathway was critical for MSCs to migrate and improve the functional restoration of SG in mice with Sjogren's-like disease[36]. Moreover, local injections of SDF-1 mobilized progenitor cells from bone marrow to the circulation and enhanced vascularisation[37]. The relationship between CD26 and angiogenesis remains controversial. There are reports that CD26 inhibited the migration of endothelial cells and formation of capillaries[38], while other reports found CD26 stimulated the proliferation and

migration of endothelial cells[39] PF4, also named as CXCL4, is a negative modulator of angiogenesis by inhibiting proliferation and migration of endothelial cells[40]. Two cytokines (moderately expressed) were also identified in the BM Soup by the cytokine microarray. IL-1 receptor antagonist (IL-1ra) is the endogenous inhibitor of IL-1. It is secreted by mononuclear cells and monocytic phagocytes[41, 42]. IL-1ra modulates IL-1 related immune and inflammation activities by blocking the IL-1 receptor. IL-1ra was reported to stimulate macrophages into a wound healing phenotype and promoting endothelial cell migration[43]. IL-16 (a CD4-binding T cell active cytokine) is a chemoattractant for CD4+ cells[44]. IL-16 can also inhibit pro-inflammatory factors, such as Th2 cytokines, IL-4, -5 and -13[45]. It was surprising to find PF4 as the highest expressed factor in BM Soup, and still this did not negatively affect the angiogenesis process in irradiated SG. Another interesting observation from our protein array results was that some previously reported growth factors involved in SG development, repair, or regeneration such as PDGF, IGF, VEGF and other FGFs (such as FGF-2 and KGF) were not detected in BM Soup. This lead us to believe that the repair of acutely irradiated SG could happen with a mixture of (growth) factors, either pro- or anti-angiogenesis, which were somehow well-orchestrated (like a cascade) during the salivary tissue repair process and this resulted in an overall improved salivary function. However, we should be warned that there are reports that injections of multipotent stem cells lead to maldifferentiation[46] and neoplasia[47]. Theoretically, BM Soup is a cell-free therapy and should possess a lesser risk to become tumorigenic. BM Soup contains fewer histocompatibility antigens, such as MHC-I and MHC-II, than the intact cells and should theoretically elicit a weaker immune response. But, since BM Soup also contain several

angiogenesis-related growth factors and cytokines (as shown in this study), additional experiments are required to investigate its effect, if any, on tumor cell growth.

Using protein arrays was a cost-effective strategy for preliminary identification of potential factor proteins in BM Soup. The limitation of the method was its relative quantification. Therefore protein arrays could not provide an exact concentration, for example, of a growth factor in BM Soup injected *in vivo* into an irradiated mouse. In future experiments, ELISA will be used to measure the actual level of each major factor in BM Soup. The second limitation of this study was that the importance of each (or a group of) identified growth factors/ cytokines in restoring salivary function remained unknown. In future studies, we will use neutralizing antibodies against selected growth factor/ cytokine to create a "specific factor-depleted BM Soup" that will be injected into irradiated mice to test their roles in the salivary gland restoration process. One last limitation: there were certainly additional (known and unknown) active protein factors in BM Soup that this study did not screen. Still, our strategy of using protein arrays facilitated the screening of BM Soup for factors and cytokines.

This preliminary screening of growth factors and cytokines released from BM Soup will assist future studies aiming to identify BM Soup-targeted genes and pathways that exert the functional restoration of irradiated SGs. These genes/ pathways can be identified by using the gene network analysis of global expression alterations caused by BM Soup treatment. We have recently used a proteomics approach to quantify 1850 proteins, based on liquid chromatography with tandem mass spectrometry (LC-MS/MS) and tandem mass tagging (TMT), for global analysis of proteins in SG cells between BM Soup-treated and untreated NOD mice[22]. Many SG proteins in Sjogren's-like disease mice were found to be

altered by the BM Soup treatment. Our future studies will use both genomic and proteomic analysis of the SG acinar/ductal cells in BM Soup-treated irradiated mice. Gene network/pathway analysis can be based on RNA-Seq data because this high-throughput technology provides a more comprehensive coverage of the genome than proteomic analysis. However, proteomics methods (such as quantitative mass spectrometry and immunoassays) are still needed because they will allow confirmation of the RNA-Seq findings at the protein level.

This study tested the benefit of BM Soup as a mitigator to minimize IR-toxicity to SG. BM Soup was injected from 5~7 days post-IR, which was during the early effect of IR-injury to SG. We selected to inject BM Soup 5 to 7 days post-IR because, based on our experience working with irradiated mice, we have noted that both the IR and the tail vein injection procedures were stressful events for the mice. Thus mice were allowed to recuperate in the animal facility center for 5 to 7 days post-IR before the first injection of BM Soup (this was our humane approach to treating IR mice). We do not know the therapeutic effect of BM Soup, if injected prophylactically, (i.e., in situations before IR therapy such as a radioprotector), or in a situation where there was already an established reduction in salivary flow (such as at 8 weeks post-IR in mice). Thus these situations remain to be tested. This study used a single fraction dose of 15 Gy, as compared to fractionated modulated radiation therapy that patients are receiving. Still our group was able to simulate the critical clinical conditions such as: 1) the irradiation was performed in clinically realistic conditions with respect to type of radiation (high-energy 6 MV photons from a clinical Varian Clinac 6EX linear accelerator applied to the salivary gland area with sufficient dose build-up), and 2) sparing of normal tissues by applying an accurately collimated slit field

with dosimetry characterized using small-field detectors[48] and with out-of-field dose well below 3%. We are currently adapting three different fractionated IR regimens, previously reported by Baum and colleagues, for the use with the Varian Clinac 6EX linear accelerator. Within these above mentioned conditions, BM Soup mitigated IR-injury by increasing saliva secretion and by protecting several cell populations in SG. Interestingly, we observed with immuno-staining of GFRα2 (used here as a marker for parasympathetic nerves) that BM Soup preserved the parasympathetic innervations while the deactivated BM Soup did not. It has been documented that parasympathetic nerve played a crucial role in SG secretion and development[49, 50]. After parasympathectomy, gland regeneration was impaired[50]. Moreover, Hai and colleagues reported that improved parasympathetic innervations restored SG function damaged by IR[51]. Our hypotheses are that: 1) some components of BM Soup protected directly the parasympathetic neurons from IR-damage, or 2) that BM Soup treatment rescued acinar, myoepithelial and endothelial cells in the SG **(Fig. 3a)**. These cells in turn secreted neurotropic factors, such as NRTN[52] and GDNF[53], which protected parasympathetic innervations from injury and promoted tissue regeneration[50]. Further investigations will be required to define these underlying mechanisms.

Materials and Methods

Preparation of BM Soup

BM Soup was prepared as described previously[21]. Briefly, each set of experiments was performed using bone marrow pooled from five 8-week old male C3H (donor) mice. BM cells were flushed from tibias and femurs in sterile PBS. The cell suspension was filtered through a 40 μm cell strainer, followed by centrifugation at 1,500 rpm for 5 min. The cell

pellet was resuspended with normal saline to a concentration of 10^7 cells/100 μl. The suspension was processed by three cycles of freeze-thaw with dry ice and a 37°C water bath. Insoluble materials were removed by micro-centrifugation at 13,500 rpm for 30 min at 4°C. The supernatant (BM Soup) was kept on ice before injections into the tail vein of recipient C3H female mice. For experiments used in this study, the BM Soup was obtained from two sets of five pooled donor mice and had a protein concentration of ~1.5 μg/μl.

Deactivation of BM Soup

Initially, four methods to deactivate BM Soup were tested. 1) Heated at 95°C for 20 min. The micro-centrifuge tube cap was wrapped with parafilm to avoid evaporation; 2) Heated at 95°C for 60 min; 3) Digested by Trypsin (1:20 w/w, T1426, Sigma-Aldrich, ST. Louis, USA) overnight at 37°C; and 4) Digested by 0.1 μg/μl proteinase K (P2308 Sigma-Aldrich, ST. Louis, USA) overnight at 37°C. Precipitates were removed by micro-centrifugation by 13,500 rpm for 15 min. 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 120V, 60 min) and Coomassie blue staining were conducted to test the efficiency of deactivation. The total protein concentration was measured by the bicinchoninic acid assay method (23225, BCA; Thermo Scientific, Pierce, IL, USA). Proteinase K digestion was the most efficient method. To safely inject into animals, 95°C heating for 20 min followed by 13,500 rpm micro-centrifugation for 15 min was performed to remove/inactivate proteinase K. The supernatant, named “deactivated BM Soup”, was kept on ice before injections into the tail vein of recipient C3H female mice.

Characterization of BM Soup and its deactivated form

The angiogenesis-related factors and cytokines within the native BM Soup and deactivated BM Soup were profiled by Proteome Profile Mouse Arrays (ARY015, ARY006, R&D Systems, Minneapolis, USA). All procedures were according to the manufacturer's instructions. Briefly, 200 µl of BM Soup, deactivated BM Soup, or normal saline was mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with Proteome Profile Mouse Array. The membranes were washed to remove the unbounded materials. Streptavidin-HRP and chemiluminescent detection reagents were used, followed by exposing membranes to X-Ray films. After subtracting background and removing the overlaps in normal saline control, the densities of signals on membranes corresponding to the amount of proteins were analyzed by Image J software (NIH).

Animals

Female C3H mice of eight weeks old purchased from Charles River (Montreal, QC, Canada) were used as recipient mice, while age-matched male C3H mice were donor mice. The mice were kept under clean condition with free food and water in animal recourse center at McGill University. All experimental procedures were performed in accordance with guidelines imposed by the Canadian Council on Animal Care and approved by the University Animal Care Committee (UACC) at the McGill University (Approved protocol #5330, www.animalcare.mcgill.ca).

Irradiation (IR)

Eight week-old female C3H mice were anesthetized with 0.3 µl/g body weight of 60 mg/ml Ketamine and 8 mg/ml Xylazine (02239093, Novopharm, Toronto, Canada) cocktail given by intra-muscular injection, and restrained in a container for irradiation. Salivary glands

were damaged by exposing them to a single 15Gy of radiation from a Varian Clinic 6EX linear accelerator (6 MV, build-up provided). The radiation was collimated to the head and neck area to guarantee less than 3% beam strength in the rest of body. After recovering from anesthesia, mice were returned to their cage and maintained in animal facility for 8 weeks post irradiation. 100 µl vehicle (normal saline), native or deactivated BM Soup was injected at 5~7 days post-irradiation, twice a week for two consecutive weeks, through the tail vein. Twenty mice were divided into 4 groups (five mice in each group): (1) Sham irradiation group (no irradiation and no injection); (2) Irradiation with injection of normal saline; (3) Irradiation with injection of BM Soup; (4) Irradiation with injection of deactivated BM Soup.

Salivary flow rate (SFR) measurement

Female mice were weighed and anaesthetized by intra-muscular injection of 0.3 µl/g body weight of a 60 mg/ml ketamine and 8mg/ml xylazine mixture. Saliva secretion was stimulated by injecting 0.5 mg/kg body weight of Pilocarpine (P6503, Sigma-Aldrich, ST. Louis, USA) subcutaneously. Whole saliva was collected into a 0.5 ml microcentrifuge tube for 10 min. The saliva volume was determined gravimetrically, assuming its density is 1 g/ml. Salivary flow rate (SFR) was determined by volume of saliva/10min/g body weight at pre-irradiation, week 4 post-IR and week 8 post-IR. At time of sacrifice (week 8 post-IR), the mice submandibular glands were harvested.

Histology staining

H&E staining.

As previously described, half of a submandibular gland was fixed in 4% PFA and embedded in paraffin. Tissue sections were cut at 5-8 μm thickness and stained by Hematoxyline and Eosin (H&E). The percentage of surface area occupied by acinar cells / total area was calculated by two independent examiners under 200X magnification of 10 fields per gland/mouse with NIH image J software

PCNA staining.

Cell proliferation staining was performed with the Zymed PCNA staining kit (931143, Invitrogen, Carlsbad, CA, USA). After deparaffinization and rehydration, five-micrometer thickness sections were blocked in 10% H_2O_2 in Methanol for 10 min. Before primary antibody labeling, tissue sections were treated three times with 10 mM Citrate Buffer solution (pH 6.1) in a 600W microwave for 5 min and then cooled down to room temperature for 30 min. Thereafter, slides were preceded with IHC routine with manufacturer's instruction. Two examiners independently counted the PCNA positive cells percentage in a blind manner under 400X magnification of 10 fields per gland/mouse with Image J software (NIH).

Blood vessel/capillary density.

Five-micrometer thickness tissue sections were stained with the antibodies to von Willebrand Factor (vWF, ECM595, Millipore Corporstion, Billerica, MA01821, USA) following the manufacturer's instruction. Briefly, after deparaffinization and rehydration, antigen retrieval was done (mentioned above). Tissue slides were incubated with rabbit anti-mouse vWF for 2 hours at room temperature. Then Goat anti-rabbit secondary

antibody was used for 15 min at room temperature. The number of blood vessels was counted under 200X magnification of 10 fields/gland with Image J software (NIH).

Immunofluorescent staining.

Frozen submandibular gland sections (6-8 μm) obtained from C3H mice were fixed in 4% PFA (P6148, Sigma-Aldrich, ST. Louis, USA) for 10 min, and then blocked in 10% normal donkey serum for 1hour to inhibit the endogenous biotin activity. These antibodies were used: (a) goat anti-aquaporin 5 (1:50, AQP5, G-19, sc-9890, Santa Cruz Biotech, Santa Cruz, CA, USA); (b) rabbit anti-alpha smooth muscle actin (1:100, α-SMA, ab7817, Abcam, Cambridge, MA, USA); (c) rabbit anti-cytokeratin 5 (1:500, CK5, Sigma-Aldrich, Oakville, ON, Canada); (d) rabbit anti-c-Kit (1:100, ab5506, Abcam, Cambridge, MA, USA); (e) goat anti GFRα-2 (1:100, R&D systems, Minneapolis, USA); Negative controls without primary antibodies were used. Sections were incubated with primary antibodies (or PBS) at 4°C overnight. After 3 times washing in PBS, slides were incubated with secondary antibodies (1:100) in the dark for 1 hour at room temperature. Secondary antibodies were donkey anti-goat- fluorescein isothiocyanate-conjugated (FITC), anti-rabbit-FITC, anti-rabbit-Alexa Fluor®594-conjugated, or anti-goat-Alexa Fluor® 594-conjugated Then, 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen, San Francisco, CA, USA) was added for 1 min to label the nucleus of cells. Fluorescence pictures were taken by Leica DM4000 fluorescent microscope. The intensity of fluorescence signal was calculated with Image J software (NIH) for at least 5 fields of 200X per gland/mouse.

Quantitative real-time PCR (qRT-PCR).

Total RNA was extracted from the mouse submandibular gland tissue with TRIZOL reagent (15596018, Invitrogen, Carlsbad, CA). 25 ng RNA per sample was used for first strand cDNA synthesis with Thermoscript RT-PCR system (11146-016, Invitrogen, Carlsbad, CA). Triplicate qRT-PCR assays were performed by Step One Plus (Life Technologies) in TaqMan Universal Master Mix II (4440040, Applied Biosystem, Foster City, Canada). The probes used were for HGF (assay ID:Mm01135193), EGF (assay ID: Mm00438696), NGF (assay ID: Mm00443039), Cyclin D1 (assay ID: Mm00432359), BMP7 (assay ID: Mm00432102), AQP5 (assay ID: Mm00437578), IGF-1R (assay ID: Mm00802841), FGF2 (assay ID: Mm00433287), MMP2 (assay ID: Mm00439498), CK5 (assay ID: Mm00503549), VEGF (assay ID: Mm01281449), GFRα2 (assay ID: Mm00433584) and TGFb1 (assay ID: Mm01178820). The expression levels of 16 endogenous control genes in mouse salivary gland tissue were first tested by TaqMan® Array Mouse Endogenous Control Plate (4426701, Life Technologies, USA). The results demonstrated that three genes, GAPDH, β-ACTN and 18sRNA, were stably expressed and unaffected by irradiation and BM Soup/ De-BM Soup treatment. Thus we selected Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, assay ID: Mm99999915) as the endogenous control gene to normalize for variations in sample input for relative quantification of gene expression in qRT-PCR experiments. Three experimental replicates were conducted for each sample. PCR was run at 50°C for 2 min, 95°C for 10 min, and 40 cycles [95°C for 15 s, 60°C for 1 min].

Statistical analysis

SPSS version 19 software was used to perform the statistical analysis. All data are presented with mean ± S.D. and were analyzed by Student's t-test or one-way ANOVA with Tukey's Post-Hoc. The statistical significance was defined as $P<0.05$.

References

1. Siegel, R., Naishadham, D. & Jemal, A. Cancer statistics, 2013. *CA Cancer J Clin* **63**, 11-30 (2013).
2. Siegel, R., Ma, J., Zou, Z. & Jemal, A. Cancer statistics, 2014. *CA Cancer J Clin* **64**, 9-29 (2014).
3. Vissink, A. *et al.* Clinical management of salivary gland hypofunction and xerostomia in head-and-neck cancer patients: successes and barriers. *Int J Radiat Oncol Biol Phys* **78**, 983-991 (2010).
4. Zheng, C. *et al.* Prevention of radiation-induced salivary hypofunction following hKGF gene delivery to murine submandibular glands. *Clin Cancer Res* **17**, 2842-2851 (2011).
5. Trotti, A. & Eisbruch, A. Reducing xerostomia through advanced technology. *Lancet Oncol* **12**, 110-111 (2011).
6. Holliday, E.B. & Frank, S.J. Proton Radiation Therapy for Head and Neck Cancer: A Review of the Clinical Experience to Date. *Int J Radiat Oncol Biol Phys* **89**, 292-302 (2014).
7. Sood, A.J. *et al.* Salivary gland transfer to prevent radiation-induced xerostomia: a systematic review and meta-analysis. *Oral Oncol* **50**, 77-83 (2014).
8. Vergeer, M.R. *et al.* Intensity-modulated radiotherapy reduces radiation-induced morbidity and improves health-related quality of life: results of a nonrandomized prospective study using a standardized follow-up program. *Int J Radiat Oncol Biol Phys* **74**, 1-8 (2009).
9. Nanduri, L.S. *et al.* Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. *Radiother Oncol* **108**, 458-463 (2013).
10. Sumita, Y. *et al.* Bone marrow-derived cells rescue salivary gland function in mice with head and neck irradiation. *Int J Biochem Cell Biol* **43**, 80-87 (2011).
11. Lombaert, I.M. *et al.* Mobilization of bone marrow stem cells by granulocyte colony-stimulating factor ameliorates radiation-induced damage to salivary glands. *Clin Cancer Res* **12**, 1804-1812 (2006).
12. Kojima, T. *et al.* Regeneration of radiation damaged salivary glands with adipose-derived stromal cells. *Laryngoscope* **121**, 1864-1869 (2011).
13. Yamamura, Y. *et al.* Treatment of salivary gland hypofunction by transplantation with dental pulp cells. *Arch Oral Biol* **58**, 935-942 (2013).
14. Anderson, D.J., Gage, F.H. & Weissman, I.L. Can stem cells cross lineage boundaries? *Nat Med* **7**, 393-395 (2001).
15. Wurmser, A.E. *et al.* Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. *Nature* **430**, 350-356 (2004).
16. Gnecchi, M., Zhang, Z., Ni, A. & Dzau, V.J. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* **103**, 1204-1219 (2008).

17. Prockop, D.J. Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. *Mol Ther* **17**, 939-946 (2009).
18. Yeghiazarians, Y. *et al.* Injection of bone marrow cell extract into infarcted hearts results in functional improvement comparable to intact cell therapy. *Mol Ther* **17**, 1250-1256 (2009).
19. Koskenvuo, J.W. *et al.* Fractionation of mouse bone-marrow cells limits functional efficacy in non-reperfused mouse model of acute myocardial infarction. *Ann Med* **44**, 829-835 (2012).
20. Angeli, F.S. *et al.* Injection of human bone marrow and mononuclear cell extract into infarcted mouse hearts results in functional improvement. *Open Cardiovasc Med J* **6**, 38-43 (2012).
21. Tran, S.D. *et al.* Paracrine effects of bone marrow soup restore organ function, regeneration, and repair in salivary glands damaged by irradiation. *PLoS One* **8**, e61632 (2013).
22. Misuno, K. *et al.* Quantitative analysis of protein and gene expression in salivary glands of Sjogren's-like disease NOD mice treated by bone marrow soup. *PLoS One* **9**, e87158 (2014).
23. Boyd, A.S. & Fairchild, P.J. Approaches for immunological tolerance induction to stem cell-derived cell replacement therapies. *Expert Rev Clin Immunol* **6**, 435-448 (2010).
24. Cotrim, A.P., Sowers, A., Mitchell, J.B. & Baum, B.J. Prevention of irradiation-induced salivary hypofunction by microvessel protection in mouse salivary glands. *Mol Ther* **15**, 2101-2106 (2007).
25. Fang, C. *et al.* An important role of matrix metalloproteinase-8 in angiogenesis in vitro and in vivo. *Cardiovasc Res* **99**, 146-155 (2013).
26. Bendeck, M.P. Macrophage matrix metalloproteinase-9 regulates angiogenesis in ischemic muscle. *Circ Res* **94**, 138-139 (2004).
27. Leen, L.L. *et al.* Estrogen-stimulated endothelial repair requires osteopontin. *Arterioscler Thromb Vasc Biol* **28**, 2131-2136 (2008).
28. Wang, Y. *et al.* Overexpression of osteopontin induces angiogenesis of endothelial progenitor cells via the avbeta3/PI3K/AKT/eNOS/NO signaling pathway in glioma cells. *Eur J Cell Biol* **90**, 642-648 (2011).
29. Patel, N., Sharpe, P.T. & Miletich, I. Coordination of epithelial branching and salivary gland lumen formation by Wnt and FGF signals. *Dev Biol* **358**, 156-167 (2011).
30. Ikari, T. *et al.* Involvement of hepatocyte growth factor in branching morphogenesis of murine salivary gland. *Dev Dyn* **228**, 173-184 (2003).
31. Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* **25**, 977-988 (2006).
32. Cheng, M. & Qin, G. Progenitor cell mobilization and recruitment: SDF-1, CXCR4, alpha4-integrin, and c-kit. *Prog Mol Biol Transl Sci* **111**, 243-264 (2012).
33. Peled, A. *et al.* Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science* **283**, 845-848 (1999).
34. Cui, L. *et al.* Stromal cell-derived factor-1 and its receptor CXCR4 in adult neurogenesis after cerebral ischemia. *Restor Neurol Neurosci* **31**, 239-251 (2013).

35. Li, S. *et al.* SDF-1alpha induces angiogenesis after traumatic brain injury. *Brain Res* **1444**, 76-86 (2012).
36. Xu, J. *et al.* Allogeneic mesenchymal stem cell treatment alleviates experimental and clinical Sjogren syndrome. *Blood* **120**, 3142-3151 (2012).
37. Kuraitis, D. *et al.* Controlled release of stromal cell-derived factor-1 for enhanced progenitor response in ischemia. *J Control Release* **152 Suppl 1**, e216-218 (2011).
38. Marchetti, C. *et al.* High mobility group box 1 is a novel substrate of dipeptidyl peptidase-IV. *Diabetologia* **55**, 236-244 (2012).
39. Kitlinska, J. *et al.* Dual role of dipeptidyl peptidase IV (DPP IV) in angiogenesis and vascular remodeling. *Adv Exp Med Biol* **524**, 215-222 (2003).
40. Kaplan, K.L., Broekman, M.J., Chernoff, A., Lesznik, G.R. & Drillings, M. Platelet alpha-granule proteins: studies on release and subcellular localization. *Blood* **53**, 604-618 (1979).
41. Dinarello, C.A. & Wolff, S.M. The role of interleukin-1 in disease. *N Engl J Med* **328**, 106-113 (1993).
42. Chamberlain, C.S. *et al.* Interleukin expression after injury and the effects of interleukin-1 receptor antagonist. *PLoS One* **8**, e71631 (2013).
43. Richmond, J., Tuzova, M., Cruikshank, W. & Center, D. Regulation of cellular processes by interleukin-16 in homeostasis and cancer. *J Cell Physiol* **229**, 139-147 (2014).
44. Little, F.F. & Cruikshank, W.W. Interleukin-16 and peptide derivatives as immunomodulatory therapy in allergic lung disease. Expert Opin Biol Ther 4, 837-846 (2004).
45. McFadden, C. et al. Preferential migration of T regulatory cells induced by IL-16. J Immunol 179, 6439-6445 (2007).
46. Breitbach, M. et al. Potential risks of bone marrow cell transplantation into infarcted hearts. Blood 110, 1362-1369 (2007).
47. Thirabanjasak, D., Tantiwongse, K. & Thorner, P.S. Angiomyeloproliferative lesions following autologous stem cell therapy. J Am Soc Nephrol 21, 1218-1222 (2010).
48. Papaconstadopoulos, P., Tessier, F. & Seuntjens, J. On the correction, perturbation and modification of small field detectors in relative dosimetry. Phys Med Biol 59, 5937-5952 (2014).
49. Knox, S.M. et al. Parasympathetic innervation maintains epithelial progenitor cells during salivary organogenesis. Science 329, 1645-1647 (2010).
50. Knox, S.M. et al. Parasympathetic stimulation improves epithelial organ regeneration. *Nat Commun* **4**, 1494 (2013).
51. Hai, B. *et al.* Transient activation of hedgehog pathway rescued irradiation-induced hyposalivation by preserving salivary stem/progenitor cells and parasympathetic innervation. *Clin Cancer Res* **20**, 140-150 (2014).
52. Xian, C.J., Huang, B.R. & Zhou, X.F. Distribution of neurturin mRNA and immunoreactivity in the peripheral tissues of adult rats. *Brain Res* **835**, 247-258 (1999).
53. Xiao, N. *et al.* Neurotrophic factor GDNF promotes survival of salivary stem cells. *J Clin Invest* **124**, 3364-3377 (2014).

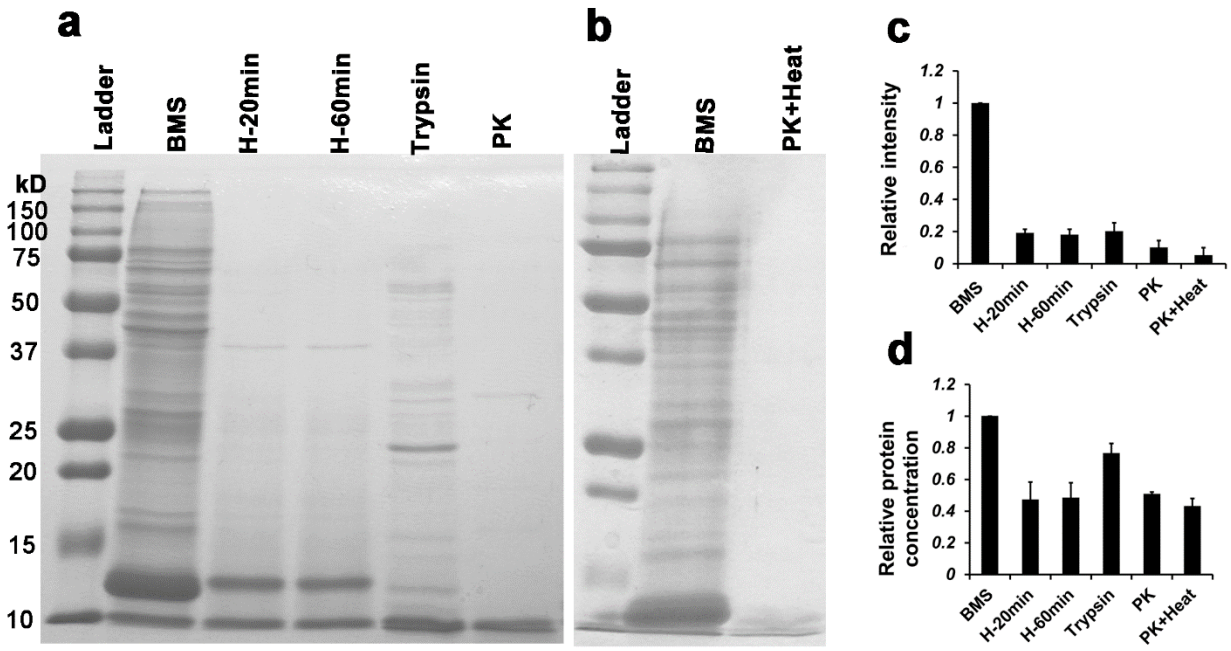

Figure 1. Deactivation of BM Soup. **(a)** Four methods to deactivate the native BM Soup (BMS) were tested, 1) BMS heated at 95°C for 20 min (H-20min); 2) heated at 95°C for 60 min (H-60min); 3) digested by trypsin (1:20 w/w) at 37°C for overnight; 4) digested by 0.1 μg/μl Proteinase K (PK) at 37°C for overnight. **(b)** Proteinase K digestion at 37°C overnight was followed by heating at 95°C for 20 min (PK+Heat) to reduce/ remove the toxicity of proteinase K when injected into live animals. Protein bands were shown by SDS-PAGE and Coomassie blue staining. **(c)** Relative quantification of proteins (intensity) on the SDS gel was analyzed with NIH Image J software. **(d)** The total protein concentration was measured by the bicinchoninic acid assay method (BCA). Three independent experiments were done. All data were normalized to BM Soup.

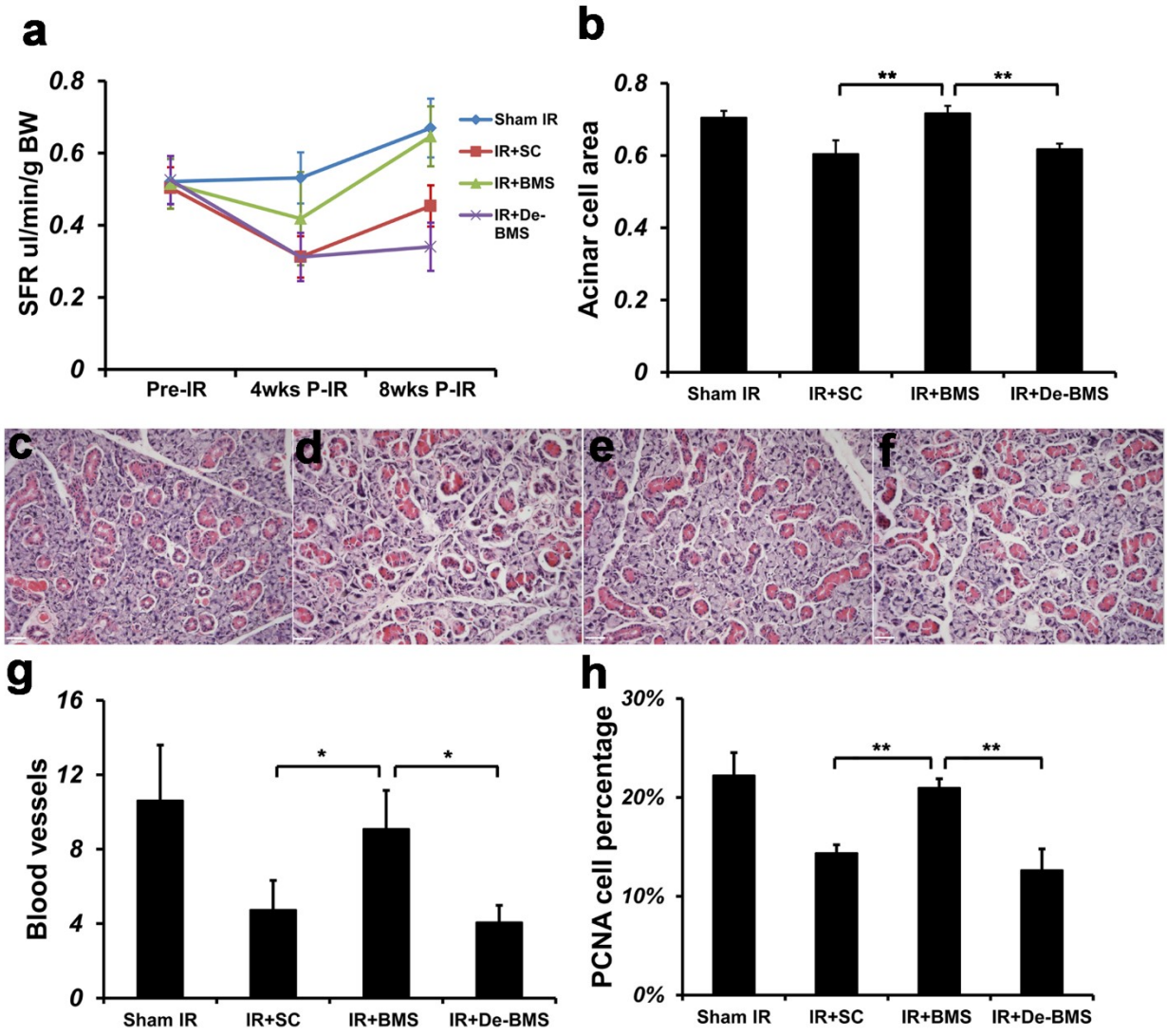

Figure 2. Role of BM Soup and 'deactivated BM Soup' on SFR, cell proliferation, acinar cells and blood vessels. (a) Salivary flow rate (SFR) was determined by volume of saliva/ 10min /g body weight at pre-irradiation (Pre-IR), 4 weeks post-IR and 8 weeks post-IR. Then mice were sacrificed and submandibular glands were harvested at 8 weeks post-IR. Histological changes were examined for **(b)** Percentage of tissue surface area occupied by acinar cells/ total area; H&E staining of mouse submandibular glands for the (**c**) Sham IR group; (**d**) Saline Control group; (**e**) BM Soup group; (**f**) Deactivated BM Soup group.

Scale bar is 38 μm. **(g)** Number of blood vessels were calculated under 200X magnification of 10 fields per gland/mouse with NIH image J software. **(h)** Percentage of PCNA positive cells was counted under 400X magnification. Two examiners independently analyzed in a blind manner. *P＜0.05; **P＜0.01, n=5 mice per group. All data were presented with mean ± S.D. BMS: BM Soup; De-BMS: deactivated BM Soup; SC: saline control.

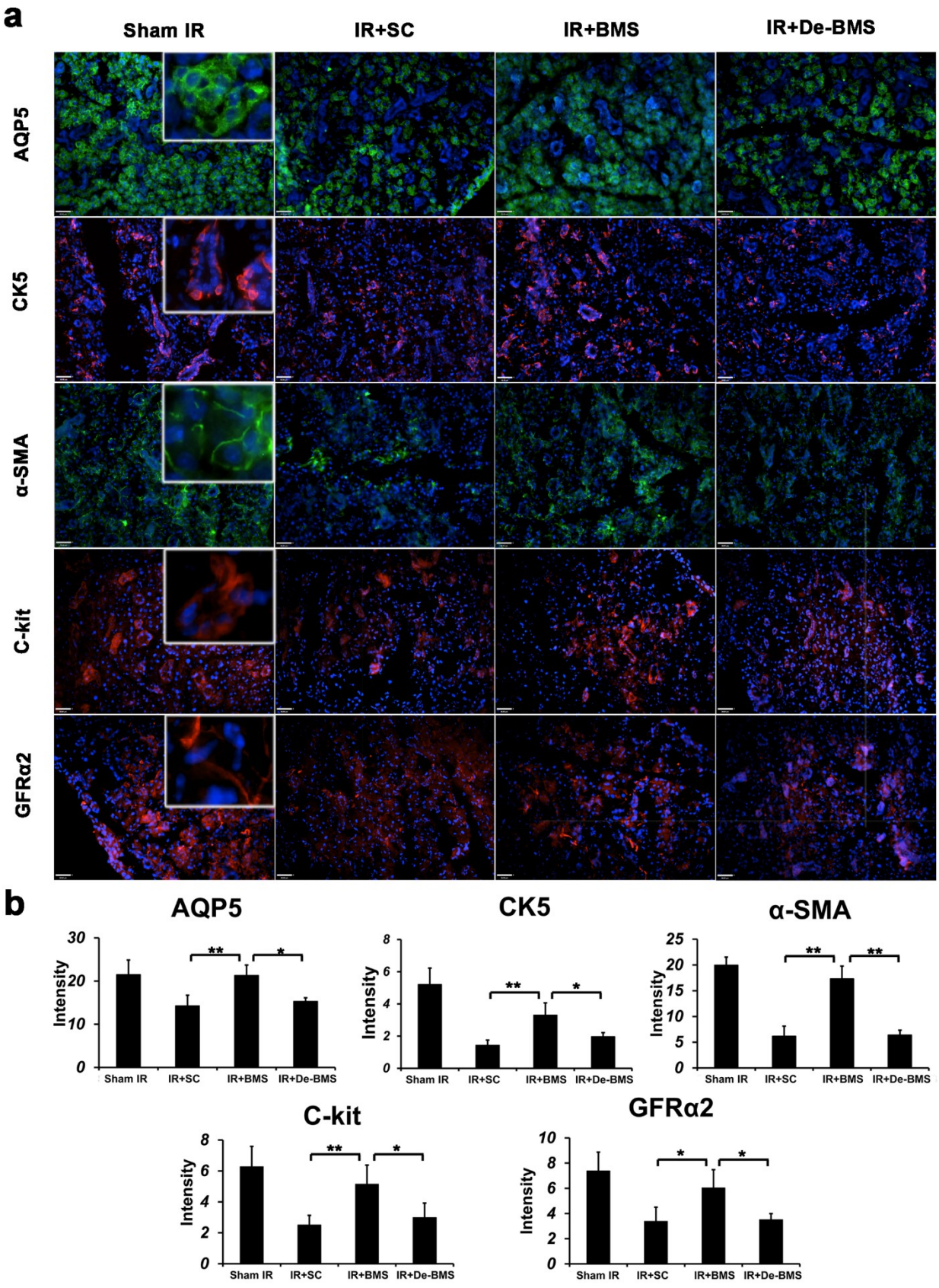
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Figure 3. Special subpopulation of gland cells was detected by immunofluorescent staining at 8 weeks post-irradiation. (**a**) The cells positive for AQP5 (marker of acinar cells), α-smooth muscle actin (marker for myoepithelial cells), CK5 (marker for ductal cells), c-Kit (marker for stem/progenitor cells), and GFRα2 (marker for parasympathetic nerves) were detected on frozen sections of salivary glands. Scale bar is 38 μm. All photographs were taken at 200X magnification. (**b**) Quantification of protein immunofluorescent expression in 5 random fields/ glands by Image J software (n= 5 mice per group; $*P<0.05$, $**P<0.01$). BM Soup group has higher protein intensity, when compared with deactivated BM Soup group. SC: saline control; BMS: BM Soup; De-BMS: deactivated BM Soup.

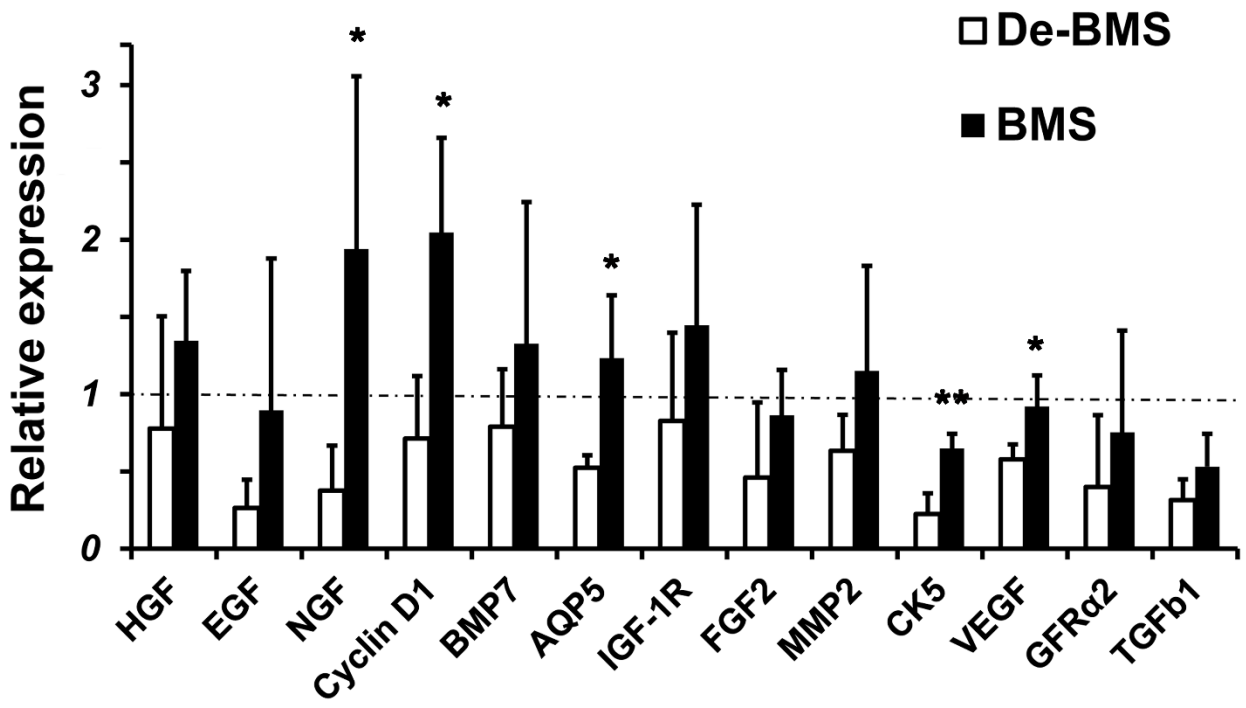

Figure 4. Relative expression of genes in salivary glands at week 8 post-irradiation. Expression levels of genes related to tissue repair/regeneration were analyzed by quantitative RT-PCR. Gene expression levels were significantly higher in BM soup-treated group than that of the deactivated BM soup for AQP5, CK5, VEGF, NGF and Cyclin D1 (cell cycle G1/Synthesis) Y-axis shows the relative expression of the gene compared to GAPDH. Horizontal dashed line represents the relative gene expression level of 1 in mice from the Control group (sham-irradiated mice). *$P<0.05$, **$P<0.01$; $n = 5$ mice. Three experimental replicates were conducted for each sample. BMS: BM Soup; De-BMS: deactivated BM Soup.

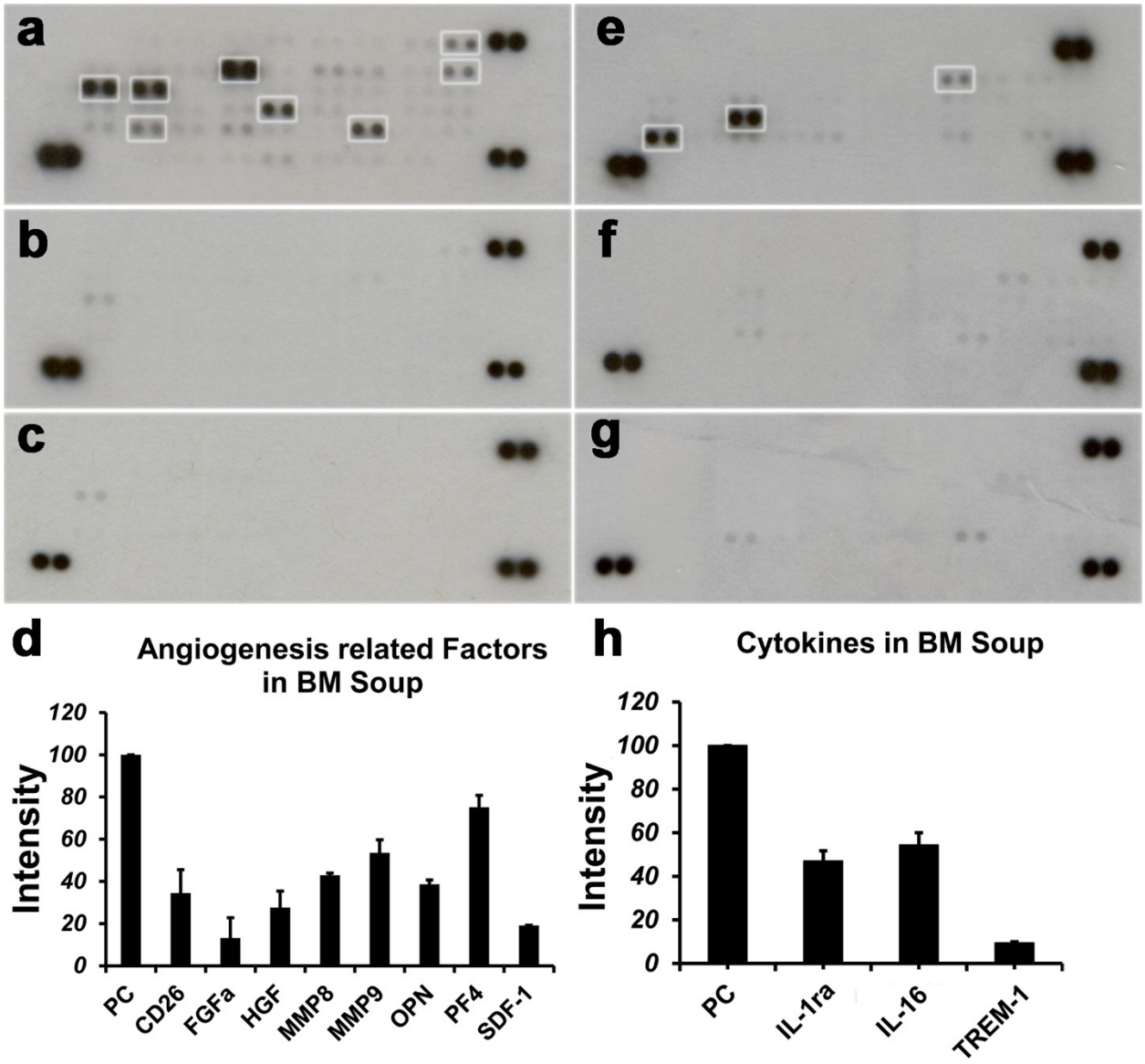

Figure 5. Characterization of BM Soup and its deactivated form. A protein microarray approach was used to characterize the angiogenesis-related growth factors in BM Soup (**a**) and deactivated BM Soup (**b**), while normal saline control (**c**) was used as a negative control. A cytokine microarray was also used to profile cytokines in the BM Soup (**e**), deactivated BM Soup (**f**) and normal saline control (**g**). (**d**) & (**h**) after background subtraction and removal of overlaps in normal saline control, quantification of factors

(intensity) was shown. Several angiogenesis-related proteins and two cytokines were identified in BM soup. After the deactivation process using proteinase K + 95°C, all these factors and cytokines were below the detection level of the two protein arrays. All data were mean ± S.D. from three independent experiments. All data were normalized to the intensity of positive control (PC, three pairs of dots at the corners).

Chapter 3-Optimal timing and frequency of Bone Marrow Soup therapy for functional restoration of salivary gland injured by single dose or fractionated irradiation

Preface

For any given therapy, the frequency and timing of treatment are the important parameters in the restoration of salivary glands. Our previous study only injected BM Soup between 5-7 days post-irradiation, which was considered to be during the early phase of IR damage to SGs. However, the optimal timing to initiate the BM Soup injection still remains unknown. In addition, animals were only followed for 8 weeks post-IR, and we expected that the therapeutic effect of BM Soup treatment decreases over time due to the degradation of its bioactive ingredients. However, whether repeated injections of BM Soup will result in the added beneficial improvements requires further investigation.

This chapter optimized the frequency and timing of BM Soup injection in mice with irradiation-damaged salivary glands. The results showed that BM Soup injections initiated between 1-3 weeks mitigate the effect of irradiation-induced injury to SGs, while delayed treatment had no saliva secretion improvement. In addition, although the therapeutic effect of BM Soup lessen after 8 weeks, it can be sustained by increasing the frequency of weekly injections.

The study presented in this chapter was submitted to the Journal of Tissue Engineering and Regenerative Medicine.

Optimal timing and frequency of Bone Marrow Soup therapy for functional restoration of salivary gland injured by single dose or fractionated irradiation

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Running Title: Optimal bone marrow soup therapy against irradiation injury

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Supported by: Canadian Institutes of Health Research, MOP-119585.

Abstract:

Injections of bone marrow (BM) cell extract, known as “BM Soup”, was previously reported to mitigate ionizing radiation (IR) injury to salivary glands (SGs). However the optimal starting time and frequency to maintain BM Soup therapeutic efficacy remains unknown. This study tests the optimal starting time and frequency of BM Soup injections in mice radiated with either a single dose or fractionated dose. First, BM Soup treatment is started at 1, 3, or 7 weeks post-IR, while positive (non-IR) and negative (IR) control mice receive injections of saline (vehicle control). Secondly, BM Soup-treated mice receive injections at different frequencies (1, 2, 3 and 5 weekly injections). Thirdly, a 'fractionated-dose radiation' model to injure mouse SGs is developed (5 Gy x 5 days) and compared to the single high dose radiation model. All mice (n = 65) are followed for 16 weeks post-IR. Results show that starting injections of BM Soup between 1-3 weeks mitigate the effect of IR-induced injury to SGs and improve the restoration of salivary function. Although the therapeutic effect of BM Soup lessen after 8 weeks, it can be sustained by increasing the frequency of weekly injections. Moreover, both single-dose and fractionated-dose radiation models are efficient and comparable in inducing SG injury, and BM Soup treatments are effective in restoring salivary function in both radiation models. In conclusion, starting injections of BM Soup within 3 weeks post-radiation, with 5 weekly injections maintain 90-100 % of saliva flow in radiated mice.

Key words: bone marrow cell extract, salivary glands, optimal timing, irradiation, head and neck cancer

Introduction

Each year, there are more than 500 000 new cases diagnosed as head and neck cancer worldwide[1]. Most of these patients undergo radiotherapy, alone or in combination with surgery and chemotherapy. Although ionizing radiation (IR) results in an improved patients' survival rate, it causes severe damage to the salivary glands (SGs). Acinar cells of SGs, although well-differentiated, are highly radiosensitive and are usually irreversibly damaged following radiation[2]. Despite new IR protocols, such as intensity-modulated radiotherapy with image guidance (IMRT/IGRT)[3] or proton radiotherapy[4], developed to spare SGs from co-irradiation damage, a substantial proportion of patients SG are still affected[5]. The diminished saliva flow contributes to numerous sequelae including dry mouth, impaired oral function, dental caries, oral mucositis and trismus; these impacting the patient's quality of life[6]. Current treatments depend on the stimulation of residual acinar cells but are generally unsatisfactory since the methods depend on re-stimulation of functional acini that are for the most part damaged[7].

Previously, our group reported that bone marrow (BM)-derived cell transplants restored saliva secretion and promoted the regeneration of irradiated SGs[8]. The exact mechanisms underlying cell-based therapy are still enigmatic. Current studies suggest that the transplanted cells participate in the tissue repair and regeneration process by releasing some specific cytokines, growth and other paracrine factors. To demonstrate this hypothesis, our group injected cell extract from whole BM cells (that we named "BM Soup"; BM Soup) into mice that had IR-injured SGs[9]. The results demonstrated that either injections of BM Soup or of whole BM cells comparably restored the function of SGs damaged by IR. This highlighted the importance of the paracrine effects of BM cells and was consistent with

studies from other organs[10]. Since BM Soup is a cell-free therapy and only contains the soluble intracellular contents, it is theoretically less tumorigenic and immunogenic than injecting intact cells[11], and this approach may be an advantage for future clinical use.

For any given therapy, the timing of its administration is an important parameter in the restoration of SG function. In a study by Lombaert et al., keratinocyte growth factor (KGF) was administered pre- and/or post-IR[12]. The results indicated that KGF ameliorated IR-induced SG damage and re-established saliva secretion to normal levels, but only when administered both pre- and post-IR. Interestingly, Limesand et al. reported that insulin growth factor (IGF-1) injected either pre- or post-IR was able to restore the function of irradiated SGs[13,14]. Different timings for MSCs treatment were also tested, such as 24h[15], 11 days[16] and as far as 10 weeks post-IR[17], which all resulted in improved SG function. From our previous study[9], BM Soup was injected between 5-7 days post-IR, which was considered to be during the early phase of IR-induced hyposalivation. However the optimal timing (therapeutic window) to initiate BM Soup therapy remains unknown, and as such can hinder the full utilization of this proposed cell-free therapy.

In addition to the timing in starting a therapy, another important parameter is the frequency (number) of injections. Injury conditions such as in myocardial infarctions[18] and in strokes[19], the timing of stem cell injections was critical, while multiple injections had no additive effects. Our group recently reported that proteins were the active ingredients in BM Soup for the restoration of SG function following IR-injury[11]. Theoretically, the therapeutic effect of the BM Soup treatment should decrease over time due to the degradation of its bioactive proteins. However, whether repeated injections of BM Soup will result in added beneficial improvements requires further investigations.

Third, animal models testing experimental therapies prefer the use of a "single dose IR" to injure SGs. The four preferred animal models of IR-induced salivary hypofunction are mice[8], rats[20], miniature pigs[21], and rhesus monkeys[22]. Using a single IR dose strategy, all these animal models had significant reductions in salivary flow and SG structural changes, which resembled hyposalivation in humans. Studies using the single dose IR is logistically easier to perform. However, in radiation therapy fractionation is introduced to allow normal tissue repair, hence, to mimic the IR delivery schedule in the clinic, a "fractionated dose IR" strategy has been successfully tested in more recent studies[23,24], and these animals demonstrated a reduced salivary flow, loss of acinar cells, and SG fibrosis[25]. Our previous BM Soup studies[9,11] only used a single dose IR regimen. It is unknown how our proposed BM Soup therapy will respond comparably when tested with the 'fractionated-dose IR' mouse model. Most mouse studies followed their experimental therapies for 8 weeks post-IR. Our study doubles that follow-up time to 16 weeks post-IR to better monitor the long-term therapeutic effect of the BM Soup therapy.

In summary, the first part of this study was aimed at testing the efficacy of the BM Soup therapy when started at different time points following IR. The second part of this study investigated the effectiveness of BM Soup injections at different frequencies. Finally, the third part of this study compared the efficacy of BM Soup in a single dose IR versus a fractionated dose IR model. Our hypothesis was that timing and frequency of BM Soup administration affect the therapeutic response in restoring salivary function. A second hypothesis was that the single dose IR model was comparable to the fractionated-dose IR model.

Materials and Methods

BM Soup preparation

As previously described[9], BM Soup was prepared from eight week-old C3H male mice. Briefly, mice were euthanized by overdose of isoflurane. Bone marrow cells were flushed out from tibias and femurs in sterile cold PBS. The cell suspension was filtered through a 40 µm cell strainer and was centrifuged at 1,500 rpm for 5 min at 4 °C. The cell pellet was resuspended in normal saline to 10^7 cells/100 µl. BM cells were frozen at -80 °C, thawed at 37 °C for three cycles, and centrifuged at 13,500 rpm for 30 min at 4 °C. The supernatant (BM Soup) was pooled to new tubes. The BM Soup was aliquoted and stored in a -80 °C until injections in the recipient C3H mice through the tail vein. In a previous study, we confirmed that frozen BM Soup retained their protein composition[11].

Animals

Eight weeks old female C3H mice (Charles River, Montreal, QC, Canada) were used as recipient mice, while the donor mice were age-matched male C3H mice. Control animals were age-matched as well. All protocols were approved by the University Animal Care Committee (UACC) at McGill University (Protocol #5330, www.animalcare.mcgill.ca).

Irradiation (IR)

Two mouse IR-injury SG models were used: a) a single dose IR or b) a fractionated IR dosage. Female C3H mice were anesthetized and restrained in a container. Salivary gland area was exposed to a single 13 Gy of radiation from a Varian Clinac 6 EX linear accelerator (6 MV). Typically, 5 mice were irradiated simultaneously with the salivary gland area under full build-up. A typical IR took 3 - 4 min. For the model using the

fractionated IR dosage, mice were firmly fixed in a restraining device without anesthesia. Salivary glands (SGs) were injured by a 5 Gy of IR per day for 5 consecutive days. At each fraction, the IR time was around 0.7 – 1.5 min depending on the fraction size. This dose was selected based on results from our pilot study testing 3, 4, 5, 6 Gy per day for 5 consecutive days. A dose of 4 Gy x 5 days appeared insufficient to reduce salivary flow, while 6 Gy x 5 days was deleterious to the health of the mice. In both single and fractionated dose models, sham-irradiated mice were used as positive controls (i.e. these mice were placed in the same setup under the linear accelerator for the same time, but without IR exposure). All mice were rehydrated with 500 µl normal saline given subcutaneously after the single dose and fractionated IR schedules.

BM Soup injections

For the timing of the BM Soup injection part, twenty mice from each radiation category were randomly divided into four groups (n = 5 mice per group; Table 1a): (1) BM Soup injections initiated at week 1 post-IR, twice a week (every Monday and Thursday morning) for two weeks; (2) BM Soup injections initiated at week 3 post-IR, twice a week (every Monday and Thursday morning) for two weeks; (3) BM Soup injections initiated at week 7 post-IR, twice a week (every Monday and Thursday morning) for two weeks; (4) vehicle control (normal saline without BM Soup) injections initiated at week 1 post-IR, twice a week (every Monday and Thursday morning) at week 1-4 and week 7 & 8. 100 µl of BM Soup or normal saline was used in each injection through the tail vein. Mice were sacrificed at week 16 post-IR, and their submandibular glands were harvested for further investigations.

For the second part of this study examining the "frequency of BM Soup injections", mice were randomized into five groups (n = 6 mice per group; Table 1b). Injections of 100 µl BM Soup was initiated one week post-IR (every Monday morning). The frequency was one injection per week and this was repeated for either 1, 2, 3 or 5 consecutive weeks (i.e., 4 'frequency treatment' groups). The negative control group consisted of 100 µl saline injected through tail vein at week 1 post-IR and once per week (every Monday morning) for 5 consecutive weeks. Mice were sacrificed at week 8 post-IR (n= 3 mice per group) and week 16 post-IR (n= 3 mice per group) to harvest their submandibular glands.

Salivary flow rate (SFR)

SFR level was measured as previously described[9]. Briefly, after anaesthetizing, saliva secretion was stimulated by injecting 0.5 mg/kg body weight of Pilocarpine (P6503, Sigma-Aldrich, St-Louis, USA) subcutaneously. Saliva was collected into pre-weighed 0.5 ml micro-centrifuge tubes for 10 min. SFR was determined by volume of saliva/ min/ g body weight. SFR was measured at week 1, 4, 8, 12, and 16 post-IR.

Periodic acid-Schiff stain (PAS)

Functional acinar cells were detected by PAS stain according to the manufacturer instructions (395B, Sigma-Aldrich, St-Louis, USA). In brief, submandibular glands were fixed in 4 % paraformaldehyde (PFA, P6148, Sigma-Aldrich, St. Louis, USA) and embedded into paraffin. Five-micrometer sections were subsequently stained with PAS reagents. The percentage of surface area occupied by acinar cells / total area was calculated by two independent examiners under 200 x magnification of 10 fields per gland / mouse with Image J software (NIH).

Cell proliferation

PCNA staining was used for cell proliferation assay (931143, Invitrogen, Carlsbad, CA, USA). After deparaffinization and rehydration, five-micrometer sections were incubated in 3 % H_2O_2 in methanol for 10 min to block the endogenous peroxidase activity. Heat induced antigen retrieval was performed at 95 °C for 10 min within 10 mM citrate buffer solution (pH 6.1) and cooled down to room temperature. Thereafter, tissue sections were proceeded with IHC following the manufacturer instructions. The PCNA positive cell percentage was counted by two examiners independently under 400 x magnification of 10 fields per gland / mouse with Image J software.

Cell apoptosis

Apoptotic activity was assessed by ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (S7100, Millipore Corporation, Temecula, CA92590, USA). Briefly, after deparaffinization and rehydration, tissue sections were incubated within 20 μg/mL proteinase K for 15 min. Then, tissue slides were incubated subsequently with terminal deoxynucleotidyl transferase (TDT) enzyme and anti-digoxigenin conjugate, and were visualized with DAB substrate. The percentage of TUNEL positive cells was counted under 400 x magnification of 10 fields / gland with Image J software (NIH).

Immunofluorescent staining

Submandibular glands were frozen in OCT and sectioned into 6-8 μm. Tissue sections were fixed in 4 % PFA for 10 min and blocked in 10 % normal donkey serum for 1 hour. Goat anti-CD31 (1:200, AF3628, R&D systems, Minneapolis, USA) was used to detect blood vessel endothelial cells. Goat anti-GFRα2 (1:200, AF429, R&D systems, Minneapolis,

USA) was used to detect parasympathetic nerves (Knox et al., 2013). PBS was used as negative control. Sections were incubated with primary antibodies (or PBS) at 4°C overnight. Tissue slides were then incubated with donkey anti-goat-fluorescein isothiocyanate-conjugated (FITC, 1:400). Then, 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen, San Francisco, CA, USA) was added for 1 min to label the nucleus of cells. Leica DM4000 fluorescent microscope and Volocity software were used to take pictures. The intensity of the fluorescence signal was calculated with Image J software for 200 x of 5 fields / glands.

Quantitative real-time PCR

Total RNA was extracted from the mouse submandibular glands with RNeasy Plus Mini Kit (74134, Qiagen, Valencia, CA91355, USA). 25 ng RNA per sample was used for first strand cDNA synthesis with High-Capacity cDNA Reverse Transcription Kit (4368814, ThermoFisher Scientific, MA02451, USA). QRT-PCR assays were performed by Step One Plus (Life Technologies) in TaqMan Universal. The probes were used for EGF (Assay ID: Mm00438696), VEGF (Assay ID: Mm01281449), IGF-1R (Assay ID: Mm00802841), AQP5 (Assay ID: Mm00437578), and GFRα2 (Assay ID: Mm00433584). Previous experiment in our lab suggested that GAPDH, β-ACTN and 18sRNA, were stably expressed and unaffected by irradiation and BM Soup treatment (Fang et al., 2015). Then, GAPDH was selected as the endogenous control gene. Three experimental replicates were performed for each sample.

Statistical analysis

Statistical analysis was conducted using SPSS version 19. One-way ANOVA with Tukey's Post-Hoc test was performed to compare the values among the groups. Statistical significance was defined as $p < 0.05$. In our study, a sample size of 3 animals is estimated to detect a 30% difference in salivary flow between BM Soup-treated and saline-treated C3H mice (alpha-level 0.05, power 0.8, mean 0.535 µl/min/g, SD 0.052 µl/min/g). Based on that calculation, a sample size of 2, 3, or 5 mice are needed to detect a 50%, 30%, or 20% difference in salivary flow rates between groups.

Results

BM Soup injections during the early phase of IR-induced hyposalivation had a more mitigating effect than when injected at a later phase.

The first part of this study tested the timing of initiating BM Soup treatment using the single dose IR mouse model. Mice that were initiated with BM Soup therapy at 1 week or at 3 weeks post-IR had a 75-85 % increase in salivary flow rate (SFR) (0.364±0.036 µl/min/g, 0.370±0.054 µl/min/g, respectively), when compared to irradiated mice injected with the vehicle control (saline) (0.201±0.059 µl/min/g, Fig. 1A; see SFR at follow-up 8 weeks post-IR; $p < 0.05$). The therapeutic efficiency lasted for 8 wks post-IR. After that time, the SFR improvement was reduced, to 50 % improvement at most (Fig. 1A; see SFR at 12 and 16 wks post-IR; $p < 0.05$). The two sets of SFR curves (from groups 'BMS wk1' and 'BMS wk3') had comparable trends and thus SFR data were superimposed in Fig 1A. Mice that were delayed in receiving the BM Soup treatment (first injections given at 7-week post-IR) had no saliva secretion improvements (0.228±0.045 µl/min/g at 8 weeks post-IR) and were thus comparable to saline-treated irradiated mice.

Acinar cells were detected histologically by PAS staining at 16 weeks post-IR. The percentage of tissue surface occupied by acinar cells was comparable in mice with BM Soup treatments initiated at either 1 week or at 3 weeks post-IR, and both of these timings had significantly higher acinar cells than the irradiated mice treated with saline (vehicle control) ($p < 0.05$; Fig. 1B, 1C). BM Soup treatments initiated at week 7 post-IR showed no improvement in acinar surface area when compared with the saline control group (Fig. 1B, 1C). Cell proliferation rate (PCNA staining) suggested the same trend as that seen with SFR and the number of acinar cells per surface area. Irradiated mice that received their first injections of BM Soup at week 1 or week 3 post-IR had a higher percentage of proliferating cells than the saline control group ($p < 0.05$; Fig. 1D). However, irradiated mice which BM Soup treatment was delayed until 7-week post-IR had a comparable percentage of proliferating cells to those in the saline control group (Fig. 1D). For cell apoptosis, there was a lower frequency in mice treated with BM Soup at 1 week and 3 weeks post-IR, with a statistically significant difference for mice treated at 1 week post-IR (Fig. 1E).

Blood vessel (CD31) density and parasympathetic innervation (GFRa2) were detected by immunofluorescent staining (Fig. 1G-J). Results suggested that BM Soup initiated between weeks 1-3 post-IR resulted in a ~2-fold higher blood vessel density in SGs of mice when compared to the saline control group (Fig. 1H; $p < 0.05$), while delayed injections showed no benefit. Similarly, parasympathetic innervations were also found a 1.5~2-fold higher in groups starting treatments at week 1-3 post-IR when compared to both the saline control and delayed injection groups (Fig. 1J). These results suggest that blood vessels and parasympathetic nerves of SGs recovered or even regenerated (because nerves were lost in

the irradiated SGs). Thus early BM Soup treatment is preferred than rather delaying it until week 7 post-IR.

At 16 weeks post-IR, the expression level of genes for factors involved in SG development/ repair (EGF, VEGF, and IGF1r), parasympathetic nerves (GFRα2) and acinar cells (AQP5) was measured by qRT-PCR (Fig. 1F). In general, all these genes were upregulated in groups with BM Soup treatment initiated between 1 week and 3 weeks. Overall, all these findings indicated the importance of early administration of BM Soup in IR-injured SGs.

Multiple injections of BM Soup maintained its therapeutic effect during a 16-week follow-up period.

The second part of the study tested the effect of injecting BM Soup at different frequencies. SGs injured with a single dose IR were treated with either normal saline or BM Soup once per week for 1, 2, 3, or 5 consecutive weeks. Repeated and frequent injections of BM Soup had no additive effect on the magnitude of the salivary secretion improvement (SFR; Fig. 2A), but this strategy provided a proportionally sustained therapeutic effect over the 16 weeks follow-up period (0.180±0.059 vs 0.295±0.021 µl/min/g, Fig. 2A, IR group versus BMS F5 group; $p<0.05$). One injection of BM Soup provided a 100 % increase in SFR when compared to the saline control group at 8 weeks post-IR, but 0 % improvement at week 16 post-IR (i.e. no sustained efficacy of the therapy) (Fig. 2A, see IR versus BMS F1 at week 8; $p < 0.05$). Mice receiving five-weekly-injections or three-weekly-injections of BM Soup had a 90-100 % and 50-80 % increase in SFR when compared to saline-injected mice, respectively, at 8 weeks post-IR and maintained these improvements until week 16 (Fig. 2A). Interestingly, at 8 weeks post-IR, all four BM Soup treated groups had

comparable SFR levels. These results indicated that repeated BM Soup injections provided a longer therapeutic effect in a frequency dependent manner.

At 8 weeks post-IR, the percentage of tissue surface occupied by acinar cell/ total area was comparable among the four BM Soup treated groups (~60 % acinar cells), which were significantly higher than the saline-treated group (43.8% ±2.49 %; $p< 0.05$; Fig. 2B, 2C). At 16 weeks post-IR, although the acinar cell surface area was less than that at 8 weeks, there was a gradual/ proportional increase of acinar cells with an increasing number of injections (Fig. 2B, 2C, $p<0.05$; note gradual increase from BMS F1 to BMS F5 at 16 wk P-IR). Mice with five-weekly-injections had a ~3-fold higher acinar cell area than the saline control group (38.2%±1.41 % versus 13.0%±1.19 %; $p<0.05$). The benefit of multiple BM Soup treatments was noted as early as 8 weeks post-IR in terms of cell proliferation and cell apoptosis (Fig. 2D, 2E). Groups with a higher injection frequency had a higher percentage of cells proliferating and a lower percentage of apoptotic cells at both 8 weeks and 16 weeks post-IR (Fig. 2D, 2E; note the dose/ frequency response effect from BMS F1 through BMS F5).

Blood vessel density and parasympathetic innervation had the same upward trend, as the one observed with SFR and acinar cells (Fig. 2G-J). That is, all four BM Soup treated groups had comparable blood vessel density and parasympathetic innervation at 8 weeks post-IR, while results at 16 weeks post-IR suggested an upward trend in the preservation of blood vessels and parasympathetic nerves in groups with an increased frequency of injections. Gene expression levels at 16 weeks post-IR was highest in the five-weekly injections, although a statistically significant difference was only found for IGF1r (Fig. 2F).

These results indicated that multiple injections of BM Soup was beneficial for the restoration of salivary function when examined over a 16 weeks following IR.

BM Soup was effective in treating salivary hypofunction in both the fractionated dose and the single dose IR mouse models.

In the last part of this study, IR was performed in a more clinically relevant strategy by fractionating the IR dose to 5 Gy per day for 5 consecutive days. Mice that received initial injections of BM Soup at week 1 or week 3 post-IR had a 50-60 % increase in SFR at the follow-up time of 8 weeks versus mice injected with the vehicle control (saline) (0.330±0.056 & 0.291±0.084 versus 0.206±0.086 µl/min/g, Fig. 3A). The first interesting finding with the fractionated dose IR model is that the therapeutic effect of the BM Soup on SFR levels was maintained during the 16-week follow-up period (Fig. 3A), while in the single dose IR model, the therapeutic effect was higher in the first 8 weeks (Fig. 1A) and then decreased between 8 to 16 weeks post-IR to SFR levels comparable to the fractionated dose IR model. The second interesting finding with the fractionated dose IR model is that SFR levels continued to decrease further at 12- and 16-week post IR when compared to the single dose IR model (a ~80 % versus a ~60 % SFR decrease, respectively; Fig. 3A). Because of this 'additional reduction' in SFR levels in the fractionated dose IR model, the BM Soup treatment at week 1 or 3 post-IR had now increased to 100 % (2-fold increase) when compared to saline controls during the (longer) follow-up time (i.e. 12 and 16 weeks post-IR). The reason for this observed increase was that the therapeutic effect on saliva flow in BM Soup-treated mice remained constant for 16 weeks while the saliva flow of saline-treated irradiated mice (negative control) decreased further at 12 and 16 weeks. As observed in the single dose IR model, BM Soup treatment started at week 7 post-IR in the

fractionated IR model ameliorated saliva output only minimally when compared to saline-treated mice.

Regarding the histological staining and gene expression levels, the fractionated dose IR mouse model had comparable results to the single dose IR model. That is, BM Soup initiated between week 1 or 3 resulted in a more acinar cell area (Fig. 3B, 3C), higher percentage of proliferating cells (Fig. 3D), reduced cell apoptosis (Fig. 3E), and a higher density of vascularization (Fig. 3G, 3H) and parasympathetic innervation (Fig. 3I, 3J) than the saline-injected control, while delaying injection of BM Soup at 7 weeks post-IR showed limited benefit. Although no statistically significant differences in gene expression levels were found in the fractionated dose IR model (Fig. 3F), the trend was comparable to the single dose IR model.

Discussion

The findings of this study were that 1) starting injections of BM Soup between 1 to 3 weeks, but not at 7 weeks post-IR, mitigated the effect of IR-induced injury to SGs and improved the restoration of salivary function; 2) although the therapeutic effect of BM Soup lessened after 8 weeks, it could be sustained by increasing the frequency of weekly injections; 3) both single-dose and fractionated-dose IR models were efficient and comparable in inducing SG injury; and 4) BM Soup was effective in restoring salivary function in both the fractionated dose and the single dose IR mouse models.

In the first part of this study, BM Soup injected during the early phase of IR-injury had more mitigating effects than when injected at a later phase. BM Soup contained numerous cytokines and growth factors[11], and these may decrease acinar cell membrane damage, apoptosis, or acute inflammation observed during the early IR-induced healing leading to

the possible hyposalivation; There is still a debate as to which event are responsible for the observed early IR-induced hyposalivation[26]. As for the reason why BM Soup did not restore (salivary) tissue function at the late-phase of IR-induced injury, we hypothesize that cytokines and growth factors contained in the BM Soup have limited (or useless) activities once acinar cells had already senesced or that their stem/progenitor cells were destroyed (sterilized) following the IR injury[27]. In a previous study, we observed an analogous restoration of salivary function when either BM or spleen cells (as an experimental therapy) were injected to mice affected with Sjögren-like (SS-like) disease[28,29]. When the therapy was given during the early phase of SS-like disease, NOD mice maintained 80-100 % of their salivary flow rate. However when the cell therapy was injected during the advanced phase of SS-like disease, only 50 % of the salivary flow rate could be re-established.

From results of the second part of this study (frequency of injections), we hypothesized that the therapeutic factors in BM Soup (proteins) were consumed or degraded during the SG remodelling/ repair process, and that multiple injections/ treatments kept the concentration of these proteins over a minimally effective level (a therapeutic threshold). Interestingly, multiple injections of BM Soup did not result in an additive effect when compared to the group of mice receiving one injection (Fig. 2A; SFR of group BMS F1 at wk 8 post-IR) but allowed to maintain the SFR gain during a longer period (Fig. 2A; SFR of group BMS F3 and BMS F5 at wk 16 post-IR). Our results provided a proof-of-concept that a long-term therapeutic effect in IR-injured SG could be achieved by increasing the frequency of BM Soup injections.

In the third part of this study, we compared two IR delivery schedules: a single dose IR versus a fractionated dose IR. High-energy 6 MV photons from a clinical Varian Clinac

6EX linear accelerator was applied to the SG area with under full build-up conditions and had an accurately collimated slit field with dosimetry characterized using small-field detectors[30] to spare the remaining surrounding normal tissues and keep the out-of-field dose well below 3 % of the central axis dose. Our results demonstrated that both IR dose models were comparable, with only a slight difference in SFR measurements toward the 12- and 16-wk follow-up time. Mice receiving either the single dose or fractionated dose IR had comparable signs observed in SGs of head and neck IR patients, including a reduced salivary flow, acinar cell atrophy, impaired vascularization and parasympathetic innervation (Supplemental Fig. S1). Both IR models had ~15-18 % acinar cell area at 16 weeks (Supplemental Fig. S1B). Saliva secretion in the single dose IR model was reduced 60 % (as compared to sham-irradiated positive controls) and remained stable during the 16-week follow-up period; while in the fractionated dose IR model, saliva secretion was further reduced to 80 % at the 12- and 16-week post-IR (Supplemental Fig. S1). Thus each IR model combined with a pre-determined follow-up time may be selected by the researchers based on their needs. For example, the "single dose IR model with an 8-week follow-up period" may be best suited when the researchers wish to initially screen the efficacy of a given experimental therapy (i.e., is the therapy effective or not?). The "single dose IR model with a 16-week follow-up" is best suited for testing the ability of an experimental therapy to maintain its efficacy (i.e., how long can the therapeutic effect be maintained?). The "fractionated dose IR model with a 16-week follow-up" is technically and logistically more challenging, and thus may be best suited for testing experimental therapies at a later stage of the validation process (such as testing efficacy and safety of an experimental therapy).

Acinar cells are the principal sites for fluid and protein secretion in saliva. It is well-documented that only a small proportion of acinar cells are lost in the early phase following IR, but the salivary flow decreases dramatically[31]. However in the late phase, it is still debatable if the remaining proportion of acinar cells is directly correlated with the quantity of saliva produced. Some studies proposed that a higher proportion of remaining acinar cells resulted in less salivary flow reduction, based on the assumption that all remaining acinar cells contributed equally to saliva secretion[32], while others debated that newly formed acinar cells could not function properly due to the damaged ductal system, blood vessels and nerves[31]. The results of this study demonstrated that mice with a higher acinar cell area remaining following IR-injury had a higher salivary flow rate, supporting the hypothesis that the number of remaining acinar cells determined the amount of saliva output in the late phase of IR injury. We do not know whether all acinar cells left were functioning properly, but more remaining acinar cells resulted in an increased saliva secretion, and that BM Soup treatments (including frequent injections) resulted in more remaining acinar cells. We have noted that the GFRa2 antibody, used as a marker for parasympathetic nerves in irradiated SGs treated with BM Soup, detected 'thicker' nerve fibers. The GFRα2-labeled parasympathetic nerves in our study looked comparable to the figures shown in the literature[33]. We hypothesize that the 'thin' nerve fibers were either damaged or difficult to be detected following irradiation, while survived 'thicker' nerve fibers were easier to detect with this GFRα2 antibody. Supplemental figure S3 shows the immunostaining of SG from normal mice with either the GFRα2 (nerve) or CD31 (blood vessel) antibody (Supplemental Fig S2).

BM Soup treatments, when compared to injections of saline, had no additional benefit nor adverse effect on the SFR of non-IR normal/healthy mice (Supplemental Fig. S3). SFR of saline- and BM Soup-injected non-IR normal mice were comparable (Supplemental Fig. S3).

Admittedly, there are limitations in this study. First, taking into account the vast number of cytokines in bone marrow cells, it will be necessary to address whether intravenous administration of cell-free extract (i.e. BM Soup) will be accompanied by a systemic inflammatory or immune response. A (pilot) supplemental short-term experiment was done by measuring serum amyloid A (SAA; an acute phase protein) and a complete blood count at 24 hours post-intravenous injection of BM Soup (Supplemental Fig S4). The results demonstrated that BMS treated mice had comparable SAA level and blood count, indicating no obvious acute inflammatory reaction was found. The long-term immune response and serum biochemistry tests will be performed in future experiments. Second, a recent study from our group found that BM Soup contained several angiogenesis-related growth factors and cytokines, such as CD26, FGF, HGF, MMP-8, MMP-9, OPN, PF4, SDF-1, IL-1ra, and IL-16[11]. We did not test whether BM Soup promoted the growth of cancer cells. Therefore, we opted to inject BM Soup only post-IR, rather than pre-IR. Additional experiments are being planned to investigate its effect on cancer cell growth. Thirdly, delayed injections of BM Soup offered no benefit for SG repair/ regeneration. For these patients, the recent strategy of delivering Aquaporin-1 cDNA using ultrasound-assisted injections may be the preferred method[34]. Fourth, BM Soup is a cell extract from whole bone marrow cells. Harvesting BM cells, either allogeneic or autologous, remains an invasive procedure. We are currently testing other types of “Cell Soup” that are easier

to be harvested clinically, such as adipose-derived mesenchymal stromal cells or peripheral blood mononuclear cells. Lastly, the exact mechanisms underlying the therapeutic effects of BM Soup remain unknown. In future experiments, both RNA-Seq and quantitative proteomics will be used to identify genes and associated pathways in SG targeted by BM Soup treatment. Once these genes and proteins are identified, the effective molecular components in BM Soup for functional restoration of damaged SGs in IR-injured mice will be validated by using a combination of immune-depletion, proteome array profiling, and animal models.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

This study was supported in part by the Canadian Institutes of Health Research (CIHR) [Grant number: MOP-119585]. We thank Dr Mari Kaartinen for access to laboratory equipment. We also thank the Center for Bone and Periodontal Research (McGill University). We also wish to thank Andre Charbonneau for his comments on the manuscript.

References

1 Baum, B. J. Radiation-induced salivary hypofunction may become a thing of the past. *Oral Dis* **22**, 81-84 (2016).
2 Cotrim, A. P., Sowers, A., Mitchell, J. B. & Baum, B. J. Prevention of irradiation-induced salivary hypofunction by microvessel protection in mouse salivary glands. *Mol Ther* **15**, 2101-2106 (2007).
3 Trotti, A. & Eisbruch, A. Reducing xerostomia through advanced technology. *Lancet Oncol* **12**, 110-111 (2011).
4 Holliday, E. B. & Frank, S. J. Proton radiation therapy for head and neck cancer: a review of the clinical experience to date. *Int J Radiat Oncol Biol Phys* **89**, 292-302 (2014).

5 Vergeer, M. R. *et al.* Intensity-modulated radiotherapy reduces radiation-induced morbidity and improves health-related quality of life: results of a nonrandomized prospective study using a standardized follow-up program. *Int J Radiat Oncol Biol Phys* **74**, 1-8 (2009).
6 Vissink, A., Jansma, J., Spijkervet, F. K., Burlage, F. R. & Coppes, R. P. Oral sequelae of head and neck radiotherapy. *Crit Rev Oral Biol Med* **14**, 199-212 (2003).
7 Khalili, S. *et al.* Mesenchymal stromal cells improve salivary function and reduce lymphocytic infiltrates in mice with Sjogren's-like disease. *PLoS One* **7**, e38615 (2012).
8 Sumita, Y. *et al.* Bone marrow-derived cells rescue salivary gland function in mice with head and neck irradiation. *Int J Biochem Cell Biol* **43**, 80-87 (2011).
9 Tran, S. D. *et al.* Paracrine effects of bone marrow soup restore organ function, regeneration, and repair in salivary glands damaged by irradiation. *PLoS One* **8**, e61632 (2013).
10 Yeghiazarians, Y. *et al.* Injection of bone marrow cell extract into infarcted hearts results in functional improvement comparable to intact cell therapy. *Mol Ther* **17**, 1250-1256 (2009).
11 Fang, D. *et al.* Identification of the active components in Bone Marrow Soup: a mitigator against irradiation-injury to salivary glands. *Sci Rep* **5**, 16017 (2015).
12 Lombaert, I. M. *et al.* Keratinocyte growth factor prevents radiation damage to salivary glands by expansion of the stem/progenitor pool. *Stem Cells* **26**, 2595-2601 (2008).
13 Grundmann, O., Fillinger, J. L., Victory, K. R., Burd, R. & Limesand, K. H. Restoration of radiation therapy-induced salivary gland dysfunction in mice by post therapy IGF-1 administration. *BMC Cancer* **10**, 417 (2010).
14 Limesand, K. H., Said, S. & Anderson, S. M. Suppression of radiation-induced salivary gland dysfunction by IGF-1. *PLoS One* **4**, e4663 (2009).
15 Lim, J. Y. *et al.* Intraglandular transplantation of bone marrow-derived clonal mesenchymal stem cells for amelioration of post-irradiation salivary gland damage. *Oral Oncol* **49**, 136-143 (2013).
16 Lin, C. Y. *et al.* Cell therapy for salivary gland regeneration. *J Dent Res* **90**, 341-346 (2011).
17 Kojima, T. *et al.* Regeneration of radiation damaged salivary glands with adipose-derived stromal cells. *Laryngoscope* **121**, 1864-1869 (2011).
18 Zhang, Y. *et al.* Timing of bone marrow cell therapy is more important than repeated injections after myocardial infarction. *Cardiovasc Pathol* **20**, 204-212 (2011).
19 Shehadah, A. *et al.* Efficacy of single and multiple injections of human umbilical tissue-derived cells following experimental stroke in rats. *PLoS One* **8**, e54083 (2013).
20 Coppes, R. P., Vissink, A. & Konings, A. W. Comparison of radiosensitivity of rat parotid and submandibular glands after different radiation schedules. *Radiother Oncol* **63**, 321-328 (2002).
21 Li, J. *et al.* Structural and functional characteristics of irradiation damage to parotid glands in the miniature pig. *Int J Radiat Oncol Biol Phys* **62**, 1510-1516 (2005).

22 Stephens, L. C. *et al.* Acute and late radiation injury in rhesus monkey parotid glands. Evidence of interphase cell death. *Am J Pathol* **124**, 469-478 (1986).
23 Cotrim, A. P. *et al.* Differential radiation protection of salivary glands versus tumor by Tempol with accompanying tissue assessment of Tempol by magnetic resonance imaging. *Clin Cancer Res* **13**, 4928-4933 (2007).
24 Xiao, N. *et al.* A novel aldehyde dehydrogenase-3 activator (Alda-89) protects submandibular gland function from irradiation without accelerating tumor growth. *Clin Cancer Res* **19**, 4455-4464 (2013).
25 Friedrich, R. E., Bartel-Friedrich, S., Holzhausen, H. J. & Lautenschlager, C. The effect of external fractionated irradiation on the distribution pattern of extracellular matrix proteins in submandibular salivary glands of the rat. *J Craniomaxillofac Surg* **30**, 246-254 (2002).
26 Vissink, A., van Luijk, P., Langendijk, J. A. & Coppes, R. P. Current ideas to reduce or salvage radiation damage to salivary glands. *Oral Dis* **21**, e1-10 (2015).
27 Pringle, S., Van Os, R. & Coppes, R. P. Concise review: Adult salivary gland stem cells and a potential therapy for xerostomia. *Stem Cells* **31**, 613-619 (2013).
28 Khalili, S. *et al.* Treatment for salivary gland hypofunction at both initial and advanced stages of Sjogren-like disease: a comparative study of bone marrow therapy versus spleen cell therapy with a 1-year monitoring period. *Cytotherapy* **16**, 412-423 (2014).
29 Khalili, S. *et al.* Bone marrow cells are a source of undifferentiated cells to prevent Sjogren's syndrome and to preserve salivary glands function in the non-obese diabetic mice. *Int J Biochem Cell Biol* **42**, 1893-1899 (2010).
30 Papaconstadopoulos, P., Tessier, F. & Seuntjens, J. On the correction, perturbation and modification of small field detectors in relative dosimetry. *Phys Med Biol* **59**, 5937-5952 (2014).
31 Konings, A. W., Coppes, R. P. & Vissink, A. On the mechanism of salivary gland radiosensitivity. *Int J Radiat Oncol Biol Phys* **62**, 1187-1194 (2005).
32 Grundmann, O., Mitchell, G. C. & Limesand, K. H. Sensitivity of salivary glands to radiation: from animal models to therapies. *J Dent Res* **88**, 894-903 (2009).
33 Knox, S. M. *et al.* Parasympathetic stimulation improves epithelial organ regeneration. *Nat Commun* **4**, 1494 (2013).
34 Wang, Z. *et al.* Ultrasound-assisted nonviral gene transfer of AQP1 to the irradiated minipig parotid gland restores fluid secretion. *Gene Ther* **22**, 739-749 (2015).

Tables:

a.	IR	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
BMS wk1	13Gy	xx	xx						
BMS wk3	13Gy			xx	xx				
BMS wk7	13Gy							xx	xx
IR Control	13Gy	xx	xx	xx	xx			xx	xx
BMS wk1	5Gy*5D	xx	xx						
BMS wk3	5Gy*5D			xx	xx				
BMS wk7	5Gy*5D							xx	xx
IR Control	5Gy*5D	xx	xx	xx	xx			xx	xx

b.	IR	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
IR Control	13Gy	x	x	x	x	x			
BMS F1	13Gy	x							
BMS F2	13Gy	x	x						
BMS F3	13Gy	x	x	x					
BMS F5	13Gy	x	x	x	x	x			

Table 1: Schedule for the start and frequency of BM Soup injections. **(a)** Timing initial injections of BM Soup. Each "x" in the table represents one injection of BM Soup (BMS) or of saline (vehicle control). "BMS wk1" means BM Soup injections initiated at week 1 post-IR. "IR Control" means injection of the vehicle control (normal saline without BM Soup). The "IR" column means irradiation at either a single dose of 13 Gy (13Gy) or 5 fractionated doses of 5 Gy daily for 5 consecutive days (5Gy*5D). "WK 1" was week 1 post-IR. (**b)** Different frequencies of BM Soup injection. "BMS F1" means one injection of BM Soup per week for one week. "BMS F2" means one injection of BM Soup per week for two consecutive weeks.

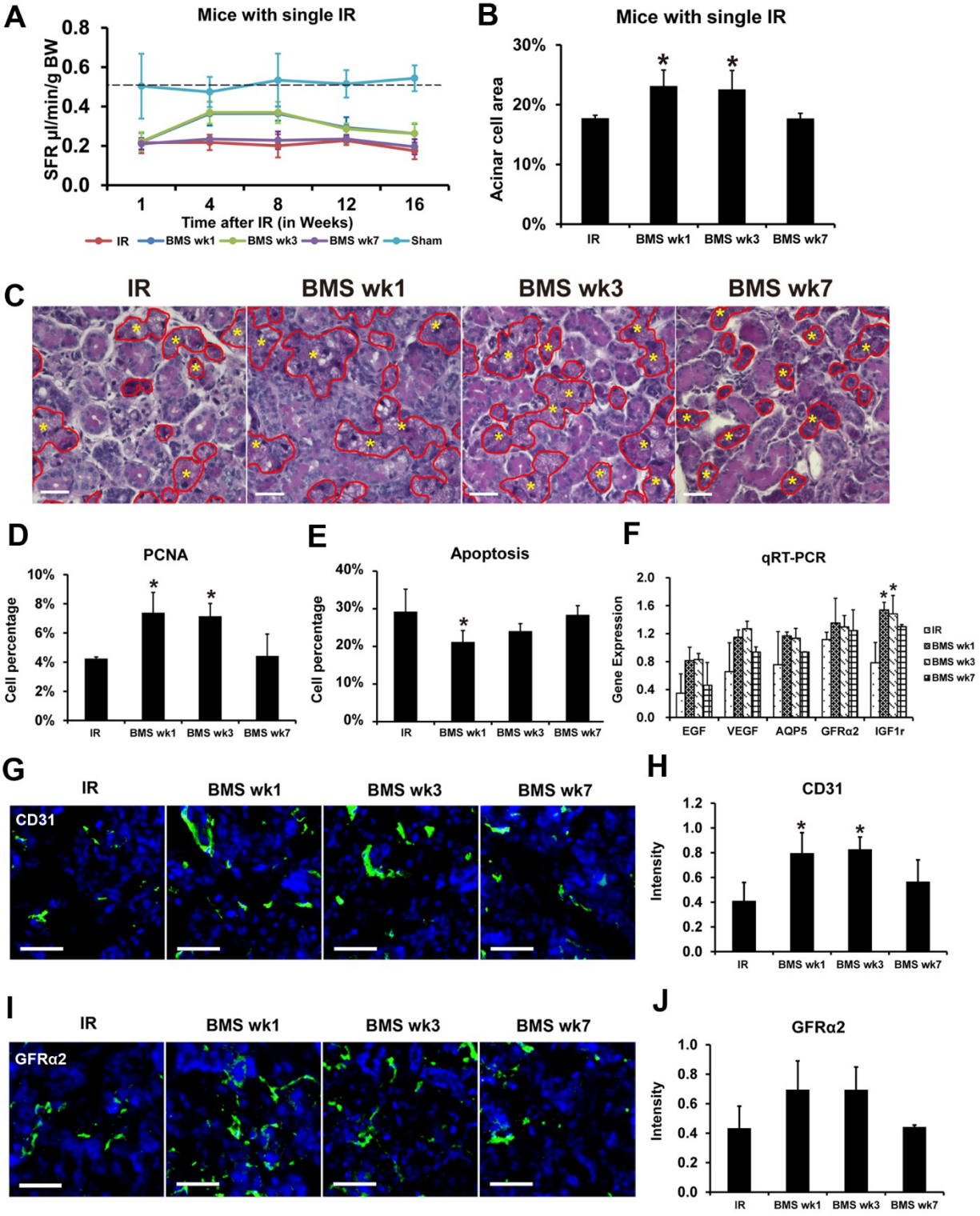

A
Mice with single IR
SFR µl/min/g BW
0.8
0.6
0.4
0.2
0.0
1
4
8
12
16
Time after IR (in Weeks)
IR
BMS wk1
BMS wk3
BMS wk7
Sham
B
Mice with single IR
Acinar cell area
30%
20%
10%
0%
IR
BMS wk1
BMS wk3
BMS wk7
C
IR
BMS wk1
BMS wk3
BMS wk7
D
PCNA
Cell percentage
10%
8%
6%
4%
2%
0%
IR
BMS wk1
BMS wk3
BMS wk7
E
Apoptosis
Cell percentage
40%
30%
20%
10%
0%
IR
BMS wk1
BMS wk3
BMS wk7
F
qRT-PCR
Gene Expression
2.0
1.6
1.2
0.8
0.4
0.0
EGF
VEGF
AQP5
GFRα2
IGF1r
IR
BMS wk1
BMS wk3
BMS wk7
G
IR
BMS wk1
BMS wk3
BMS wk7
CD31
H
CD31
Intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
IR
BMS wk1
BMS wk3
BMS wk7
I
IR
BMS wk1
BMS wk3
BMS wk7
GFRα2
J
GFRα2
Intensity
1.0
0.8
0.6
0.4
0.2
0.0
IR
BMS wk1
BMS wk3
BMS wk7

Fig. 1: Timing of BM Soup injections for functional restoration of salivary function. **(A)** Time course of salivary flow rate (SFR) measurements (shown as µl of saliva/ per minute/ per gram of body weight) of mice irradiated at 13 Gy. BM Soup injections were initiated at either wk 1 post-IR (group BMS wk1), wk 3 (group BMS wk3), or wk 7 (group BMS wk7). The group "IR" means irradiated mice injected with normal saline (i.e. the vehicle control without BM Soup). The "Sham" group means that mice were placed in the same setup under the linear accelerator for the same length of time, but did not receive IR exposure. The horizontal dash-line represents the baseline pre-irradiation SFR level of normal/healthy age-matched mice. N= 5 mice per group. All data were presented with mean ± S.D. * $p < 0.05$). Please note that the two sets of SFR curves (from groups 'BMS wk1' and 'BMS wk3') had comparable trends and thus their SFR data were superimposed in the graph. **(B)** Percentage of tissue surface area occupied by acinar cells. **(C)** Acinar cells were detected by PAS stain. Acini surfaces are outlined in red with yellow stars. Scale bars in panels c, g, i are all 38 µm. **(D)** The percentage of PCNA positive cells (cell proliferation) and **(E)** TUNEL positive cells (cell apoptosis). **(F)** Gene expression level for factors involved in salivary gland development/repair (EGF, VEGF, and IGF1r), parasympathetic nerves (GFRα2) and acinar cells (AQP5) tested by qRT-PCR. Y-axis shows the relative expression of the gene compared to GAPDH. Three experimental replicates were conducted for each sample. **(G, H)** Cells positive for CD31 (marker for blood vessel endothelial cells) and **(I, J)** GFRα2 (marker for parasympathetic nerves) detected by immunostaining and their expression was semi-quantified by their fluorescence intensity in 5 random fields/glands. Overall, these results indicated that BM Soup injections

during the early phase of IR-induced hyposalivation had a more mitigating effect than when injected at a later phase.

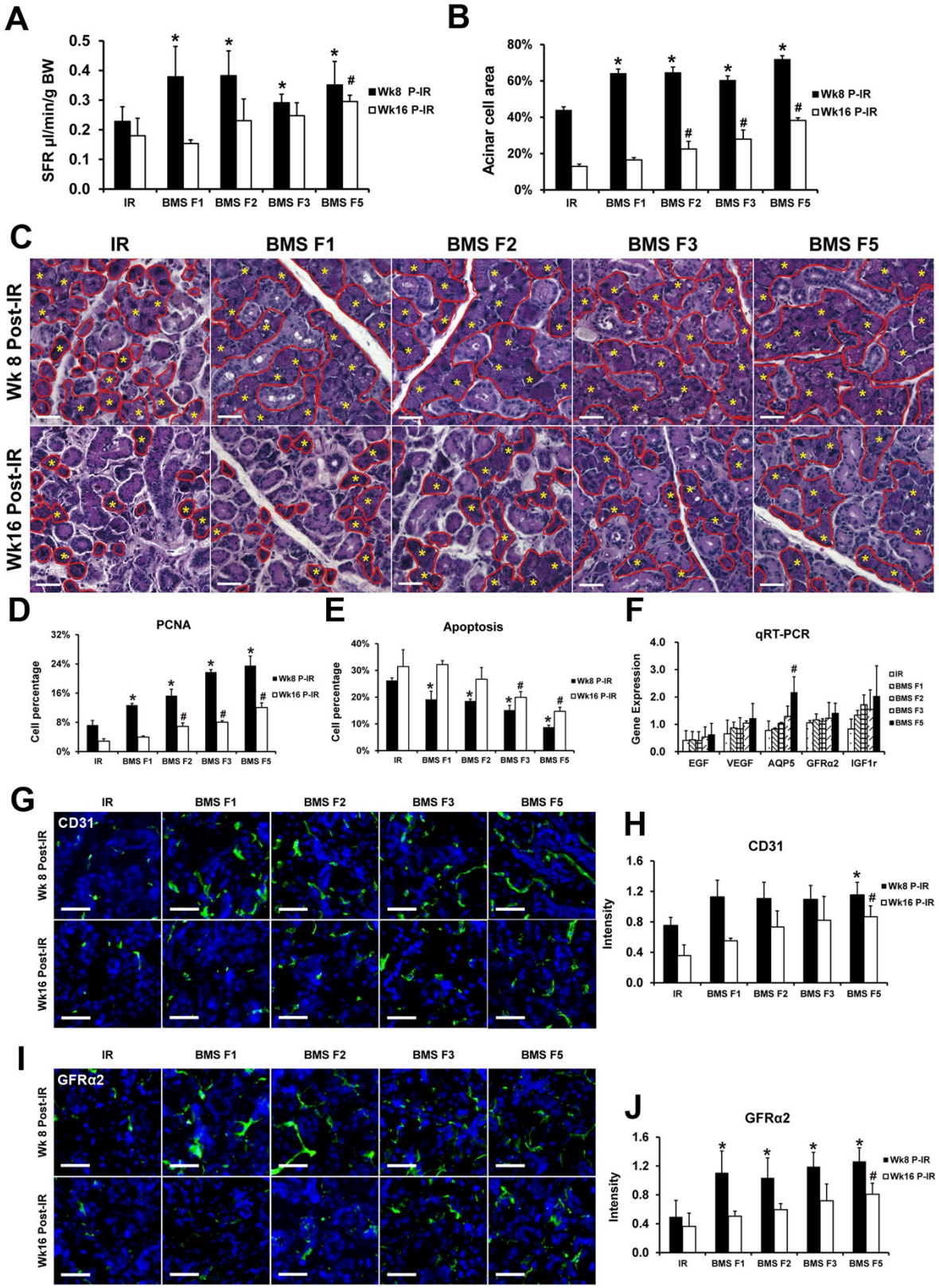

A
SFR µl/min/g BW
0.5
0.4
0.3
0.2
0.1
0.0
IR
BMS F1
BMS F2
BMS F3
BMS F5
Wk8 P-IR
Wk16 P-IR
B
Acinar cell area
80%
60%
40%
20%
0%
C
IR
BMS F1
BMS F2
BMS F3
BMS F5
Wk 8 Post-IR
Wk16 Post-IR
D
PCNA
Cell percentage
32%
24%
16%
8%
0%
E
Apoptosis
40%
30%
20%
10%
0%
F
qRT-PCR
Gene Expression
4.0
3.0
2.0
1.0
0.0
EGF
VEGF
AQP5
GFRα2
IGF1r
G
CD31
H
Intensity
1.6
1.2
0.8
0.4
0.0
I
GFRα2
J

Fig. 2: Frequency of BM Soup injections for restoration of salivary function at 8 and 16 weeks post-irradiation. (A) Salivary flow rate (SFR) measurements at 8 weeks (black) and 16 weeks post-IR (white) in mice injected with an increased frequency of BM Soup. "BMS F1, F2, F3, or F5" means one injection of BM Soup per week for one, two, three or five weeks, respectively. "IR" were irradiated mice injected with normal saline (i.e. the vehicle control without BM Soup). n= 3-5 mice per group. All data were presented with mean ± S.D. * $p < 0.05$ when compared to IR group at week 8 post-IR. # $p < 0.05$ when compared to IR group at week 16 post-IR. **(B)** Percentage of tissue surface area occupied by acinar cells at 8 and 16 wk post-IR. **(C)** Acinar cells detected by PAS stain. Acini surfaces are outlined in red with yellow stars. Scale bars in panels c, g, i are all 38 μm. **(D)** PCNA positive cells and **(E)** TUNEL positive cells. **(F)** At week 16 post-IR, gene expression level relative to GAPDH. **(G, H)** Immunofluorescence staining and semi-quantitative expression of CD31 (endothelial cell marker) and **(I, J)** GFRα2 (parasympathetic nerve marker). Overall, these results indicated that although the therapeutic effect of BM Soup lessened after 8 weeks, it could be sustained by increasing the frequency of weekly injections. A frequency of 5 weekly injections maintained the efficacy of BM Soup treatment for 16 weeks post-IR.

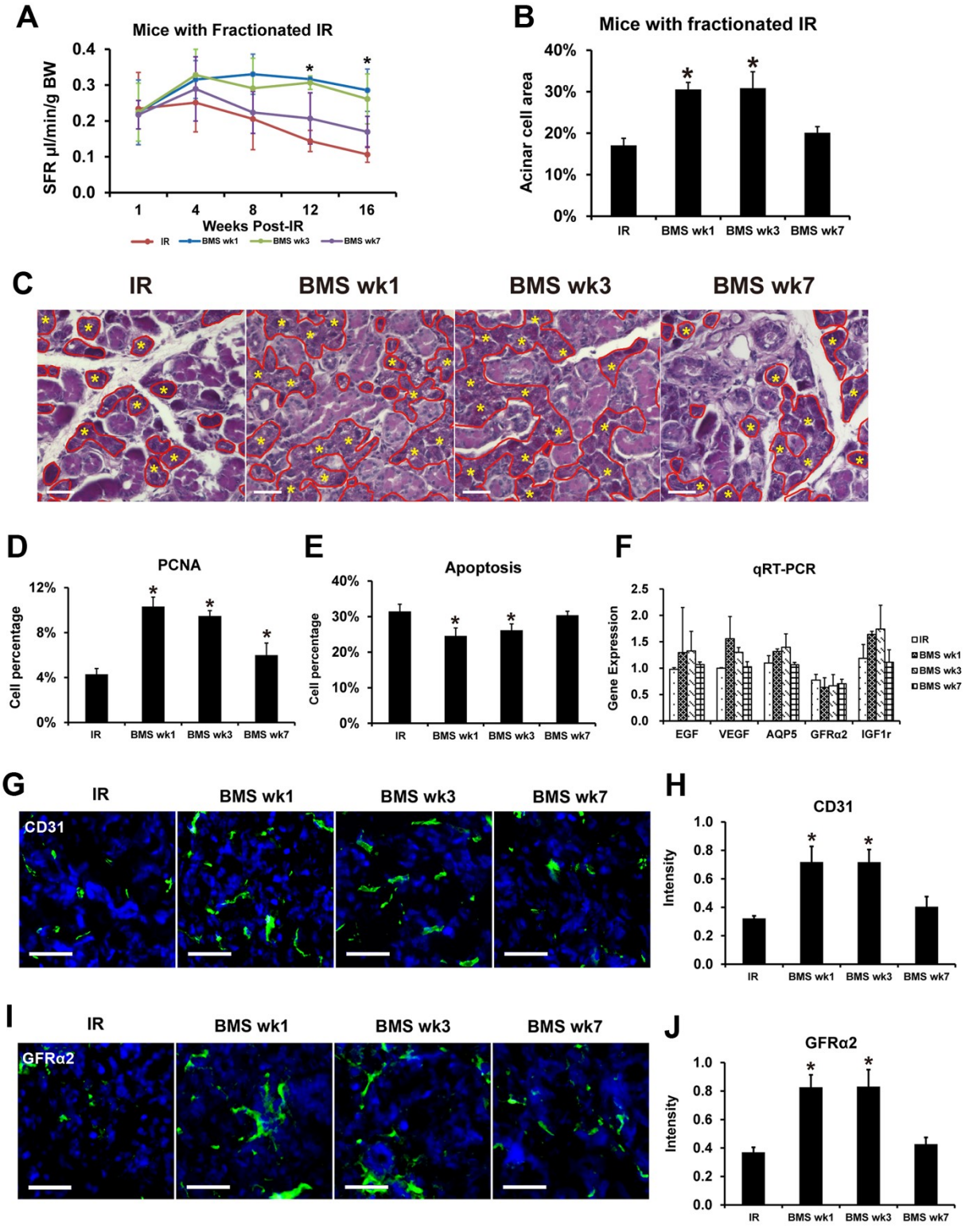
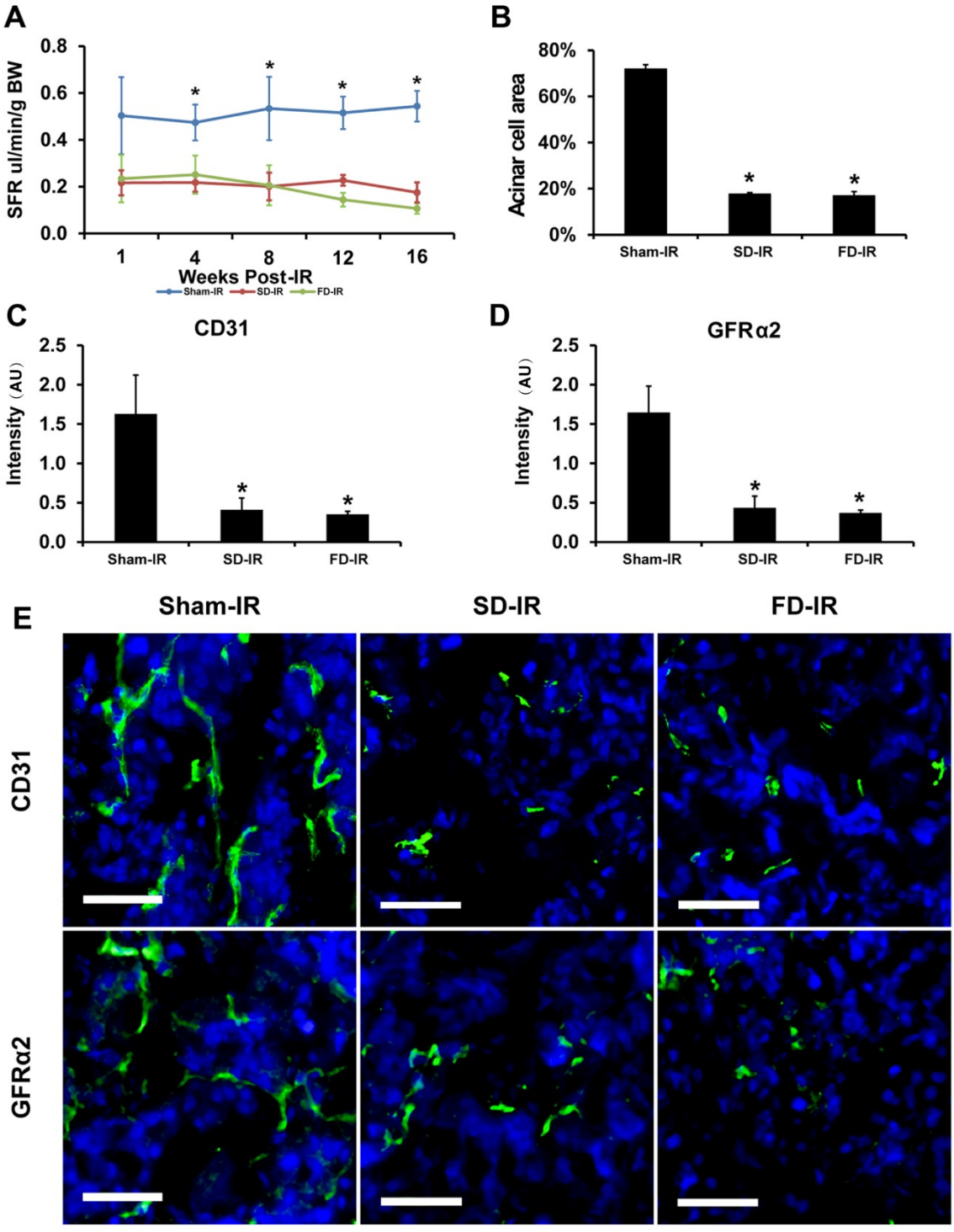
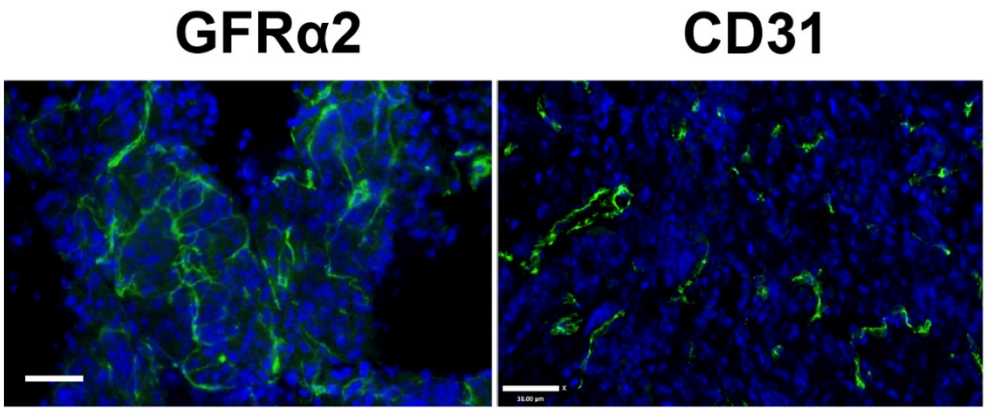

A
Mice with Fractionated IR
SFR µl/min/g BW
0.4
0.3
0.2
0.1
0.0
1
4
8
12
16
Weeks Post-IR
IR
BMS wk1
BMS wk3
BMS wk7
B
Mice with fractionated IR
Acinar cell area
40%
30%
20%
10%
0%
IR
BMS wk1
BMS wk3
BMS wk7
C
IR
BMS wk1
BMS wk3
BMS wk7
D
PCNA
Cell percentage
12%
8%
4%
0%
IR
BMS wk1
BMS wk3
BMS wk7
E
Apoptosis
Cell percentage
40%
30%
20%
10%
0%
IR
BMS wk1
BMS wk3
BMS wk7
F
qRT-PCR
Gene Expression
2.5
2.0
1.5
1.0
0.5
0.0
EGF
VEGF
AQP5
GFRα2
IGF1r
IR
BMS wk1
BMS wk3
BMS wk7
G
IR
BMS wk1
BMS wk3
BMS wk7
CD31
H
CD31
Intensity
1.0
0.8
0.6
0.4
0.2
0.0
IR
BMS wk1
BMS wk3
BMS wk7
I
IR
BMS wk1
BMS wk3
BMS wk7
GFRα2
J
GFRα2
Intensity
1.0
0.8
0.6
0.4
0.2
0.0
IR
BMS wk1
BMS wk3
BMS wk7

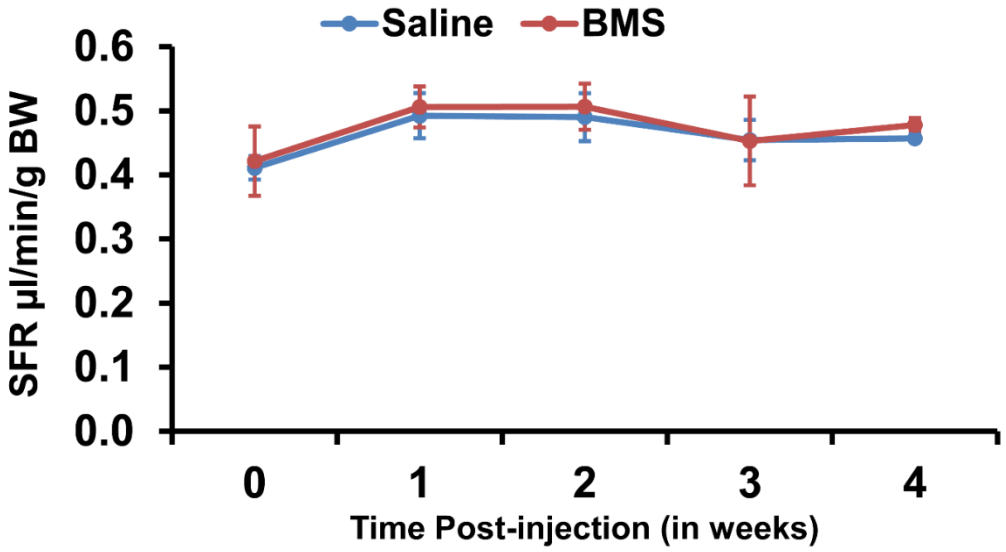
Fig. 3: Efficacy of BM Soup when tested in the fractionated dose IR mouse model. (A) Time course of SFR measurements (μl per min per gram of body weight) of mice irradiated at 5 Gy daily for 5 consecutive days. BM Soup injections were started at wk 1 post-IR (BMS wk1), wk 3 (BMS wk3), or wk 7 (BMS wk7). "IR" were irradiated mice injected with normal saline (i.e. the vehicle control without BM Soup). n= 5 mice per group. All data were presented with mean ± S.D. * $p < 0.05$). **(B)** Percentage of tissue surface area occupied by acinar cells. **(C)** Acinar cells detected by PAS stain. Acini surfaces are outlined in red with yellow stars. Scale bars in panels c, g, i are all 38 μm. **(D)** The percentage of PCNA positive cells (cell proliferation) and **(E)** TUNEL positive cells (cell apoptosis). **(F)** At week 16 post-IR, gene expression level relative to GAPDH. **(G, H)** Immunofluorescence staining and semi-quantitative expression of CD31 (endothelial cell marker) and **(I, J)** GFRα2 (parasympathetic nerve marker).


A
SFR ul/min/g BW
0.8
0.6
0.4
0.2
0.0
1
4
8
12
16
Weeks Post-IR
Sham-IR
SD-IR
FD-IR
B
Acinar cell area
80%
60%
40%
20%
0%
Sham-IR
SD-IR
FD-IR
C
CD31
Intensity (AU)
2.5
2.0
1.5
1.0
0.5
0.0
Sham-IR
SD-IR
FD-IR
D
GFRα2
Intensity (AU)
2.5
2.0
1.5
1.0
0.5
0.0
Sham-IR
SD-IR
FD-IR
E
Sham-IR
SD-IR
FD-IR
CD31
GFRα2

Supplemental Fig S1: Efficacy of BM Soup therapy in the single dose versus the fractionated irradiation mouse models. (A) Time course of SFR measurements of mice irradiated with either a 13 Gy single dose irradiation (SD-IR) or a fractionated irradiation dose of 5 Gy x 5 days (FD-IR). "Sham-IR" mice were placed inside the linear accelerator but no IR was actually delivered and were thus used as positive controls because these mice had normal salivary flow. n= 5 mice per group. All data were presented with mean ± S.D. * $p < 0.05$) when compared to Sham-IR group. **(B)** Percentage of tissue surface area occupied by acinar cells. **(C, E)** Immunofluorescence staining and semi-quantitative expression of CD31 (endothelial cell marker) and **(D, E)** GFRα2 (parasympathetic nerve marker). Scale bar= 38 μm.

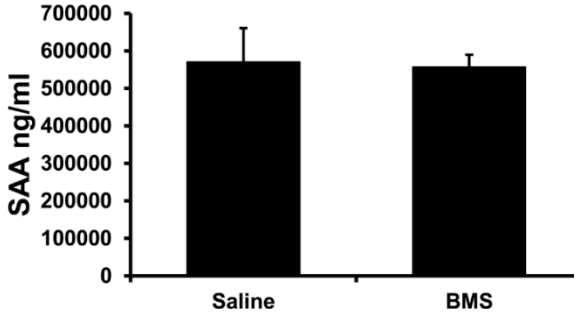


Supplemental Fig S2: Immunostaining of SG from normal mice with either the GFRα2 or CD31 antibody. GFRα2 was used as a marker to detect parasympathetic nerves, while CD31 as a marker for blood vessel endothelial cells. Scale bar=38 μm.



Supplemental Fig. S3: No effect of BM Soup on salivary gland of normal mice (sham-IR mice). 100 µl of saline or BM Soup was injected through the tail vein of sham-IR (no-IR) healthy mice and their salivary flow rate (SFR) was measured once a week for 4 weeks. BM Soup treatment had no additional benefit or adverse effect on SFR of normal mice. All data were presented with mean ± S.D, (n= 3 mice).

A



B

	Saline		BMS	
	Mean	SD	Mean	SD
WBCs x 10^9/L	3.2	0.0	2.3	0.8
RBCsx 10^{12}/L	7.1	0.0	7.5	0.6
hemoglobin g/L	139.0	1.4	146.0	11.3
hematocrit L/L	0.4	0.0	0.4	0.0
MCV fL	52.0	0.0	52.0	0.0
MCH pg	19.5	0.2	19.4	0.1
MCHC g/L	375.0	4.2	372.0	1.4
platelets x 10^9/L	366.5	113.8	616.0	149.9
neutrophils %	24.0	5.7	21.0	4.2
Lymphocytes %	75.5	6.4	78.0	2.8
monocytes %	0.0	0.0	0.0	0.0
eosinophils %	0.5	0.7	1.0	1.4

Supplemental Fig. S4: No obvious acute inflammatory reaction at 24 hours after BM Soup injection. (A) Serum amyloid A (SAA; an acute phase protein) was measured as a marker for acute inflammation and **(B)** complete blood count was performed at 24 hours after BM Soup injection in normal mice. The results demonstrated that BMS treated mice had comparable SAA level and blood count, indicating no obvious acute inflammatory reaction was found (n=2 mice).

Chapter 4- Cell extracts/ soups from spleen and adipose tissues restores function to irradiation-injured salivary glands.

Preface

In the previous study, our group reported that BM Soup was able to restore the function of salivary gland (SG) and improve the tissue repair and regeneration following local irradiation. As a cell-free therapy, BM Soup only contains soluble intracellular contents and is theoretically less tumorigenic and immunogenic when compared with the intact cells. However, BM cell harvesting remains an invasive procedure and could lead to donor discomfort, and in some severe cases, to life-threatening complications, such as cardiopulmonary problems, bacterial infections, and cerebrovascular accidents.

In this Chapter, we tested the therapeutic effects of cell soups from adipose-derived stromal cells (ADSCs) and spleen cells to repair salivary glands. BM Soup, Spleen Soup, or ADSC Soup was injected into mice with IR-injured SGs (13Gy). Irradiated mice with saline (vehicle) injection served as negative control. Results demonstrated that all three cell soups restored saliva production, protected acinar cells, blood vessels, and parasympathetic nerves, and increased cell proliferation. Moreover, although protein array assays found more angiogenesis-related growth factors in ADSC Soup, BM Soup and Spleen Soup maintained the therapeutic effect for a longer time than the ADSC Soup.

The study presented in this chapter is in preparation for publication.

Cell extracts/ soups from spleen and adipose tissues restores function to irradiation-injured salivary glands.

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Running Title: Adipose and spleen cell soups mitigate radiation-injury to salivary glands

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Email: simon.tran@mcgill.ca

Supported by: Canadian Institutes of Health Research, MOP-119585.

Abstract

A cell extract from whole bone marrow (BM), which we named "BM Soup", has the property to restore saliva secretion to irradiation (IR)-injured salivary glands (SGs). However, BM cell harvesting remains an invasive procedure for the donor. The main objective of this study was to test the therapeutic effect of "Cell soups" obtained from alternate tissues, such as adipose-derived stromal cells (ADSCs) and spleen cells to repair SGs. BM Soup, Spleen Soup, ADSC Soup, or saline (vehicle control) was injected intravenously into mice with IR-injured SGs (13Gy). Results demonstrated that all three cell soups restored saliva production, protected acinar cells, blood vessels, and parasympathetic nerves, and increased cell proliferation. While protein array assays identified more angiogenesis-related growth factors in ADSC Soup, the length of its therapeutic efficiency on saliva flow was less than that of the BM Soup and Spleen Soup. Another objective of this study was to compare "Fresh" versus "Cryopreserved (-80°C)" BM Soup. It was found that the therapeutic effect of twelve-month "Cryopreserved BM Soup" was comparable to that of "Fresh BM Soup" on the functional restoration of IR-injured SGs. In conclusion, both Spleen Soup and ADSC Soup can be used to mitigate IR-damaged SGs.

Introduction

It is estimated that 48,000 new patients are diagnosed with oral-pharyngeal cancer, and around 10,000 patients die due to this disease each year in the United States [1]. Radiotherapy is a commonly used curative treatment for these patients. However, irradiation can inevitably injure the healthy tissue surrounding the tumor and cause severe side effects, such as salivary hypofunction[2]. The reduced saliva flow predisposes the patients to numerous sequelae, including dryness of the mouth, difficulties in oral functioning, radiation caries, and oral mucositis, which impact the patient's quality of life[3]. Unfortunately, current clinical treatments remain palliative, and no restorative therapy is available.

In a previous study, our group reported that a bone marrow cell extract, namely 'BM Soup', was able to restore the function of salivary gland (SG) following head and neck irradiation, and improved tissue repair and regeneration [4,5]. As a cell-free therapy, BM Soup only contains soluble intracellular contents and is theoretically less tumorigenic and immunogenic when compared with intact (while) cells[5]. A study by Angeli FS et al. demonstrated that local injection of BM Soup originated from human resulted in functional improvement of infarcted hearts in an immune-competent mouse model[6]. Thus, BM Soup treatment is not patient-specific and can be performed without requiring histocompatible donors. However, bone marrow cell harvesting procedure remains invasive and leads to donor discomfort[7] and in severe cases, to life-threatening complications such as cardiopulmonary problems, bacterial infections and cerebrovascular accidents[8]. Therefore, the primary objective of this study was to test if other types of tissues, which were either clinically easier to harvest (such as the adipose tissue) or regarded as 'dispensable organ'

(such as the spleen), could be used as alternate sources of "Cell Soup" for the repair of IR-injured salivary glands.

Our first alternate tissue is the adipose-derived stromal cell (ADSC). Like bone marrow mesenchymal stem cells (BMMSCs), ADSCs are multipotent and can be differentiated into adipocytes, osteoblasts, and chondrocytes[9]. Moreover, current studies have demonstrated that systemic injections of ADSCs ameliorated SG damage induced by irradiation and improve salivary secretion[10-12]. In addition, the therapeutic effects of ADSCs on tissue regeneration were mostly attributed to the bioactive secretomes produced by the cells[13-14], including growth factors, cytokines and extracellular vesicles, and these paracrine factors were also suggested to be responsible for the mitigation of IR damage to SGs[11]. Based on the above studies, we hypothesized that ADSC Soup which contains numerous bioactive secretomes will restore salivary function following IR, and that ADSC Soup can serve as a substitute for BM Soup. Advantages of using ADSC Soup are that ADSCs can be harvested with less invasive procedures, such as from resected adipose tissue or liposuction.

Spleen has been considered as an unnecessary organ by conventional medical wisdom[15]. Recently, it was reported to be a source of unique stem cell population and gained an increased attention in regenerative medicine[16,17]. Multipotent stem cells from spleen were found to restore the function of several tissues or organs, including pancreatic islets[18], cranial nerves[19], hearts[20], and inner ear[19]. Moreover, the spleen is the supplementary for BM to form the blood cells. Previously, our group injected splenocytes (spleen cells) combined with complete Freund's adjuvant (CFA) to treat Sjögren's-like disease, an autoimmune disease leading to salivary hypofunction, in the NOD mouse model[21]. The results demonstrated that splenocytes and BM cells resulted in a comparable improvement

of salivary function [22]. In this study, we hypothesized that the therapeutic effect of the cell extract from splenocytes, namely the "Spleen cell soup" would be comparable to that of the BM Soup for the restoration of salivary function in IR-injured SGs.

In addition, the patients with radiotherapy cannot afford the invasive procedure to harvest fresh BM cells. Thus, autologous BM Soup should be prepared pre-IR. Then, before injected post-IR., it requires at least 2 months storage during the radiotherapy is performed, and even longer time if the surgery and/or chemotherapy is combined. Moreover, BM Soup treatment needs repeated injections and some cases might require additional injections long after the first time. Even if the allogeneic BM Soup is used, repeated isolation of fresh BM cells remains an ethical issue. Therefore, long-term safe preservation is important for the clinical application of the BM Soup, either autologous or allogeneic. Thus, another objective of this study was to test if BM Soup can be simply cryopreserved in -80°C freezer for long-term storage.

In summary, the first purpose of this study was to compare the efficacy of different cell soups and investigated which cell soup could best restore the function of IR-injured SG. Secondly, we aimed at testing whether BM Soup can be stored in -80°C freezer for 12 months.

Materials and Methods

Animals

Eight-week-old male C3H mice (Charles River, Montreal, QC, Canada) were used as a donor for bone marrow cells, spleen cells, and adipose-derived stromal cells, while age-matched female mice as the recipient. Animals were kept in clean condition with free food

and water in the animal resource center at McGill University. All procedures were performed under protocols approved by the University Animal Care Committee (UACC) at McGill University (Protocol #5330, www.animalcare.mcgill.ca).

Preparation of Cell Soups

BM Soup was prepared from five male mice of eight-week-old as described previously[5]. Briefly, bone marrow cells were flushed out from tibia and femurs in sterile cold PBS. The cell suspension was filtered through a 40 µm cell strainer and centrifuged at 400 g for 5 min at 4 °C. Cell pellet was resuspended in normal saline at the concentration of 10^7 cells/100 µl. Cell solution was frozen at -80 °C, thawed at 37 °C for three cycles, followed by centrifugation at 17,000 g for 30 min at 4 °C. The supernatant (BM Soup) was pooled into a new tube and then was stored in a -80 °C freezer until injections into the recipient mice.

Spleen tissues harvested from male C3H donor mice were mashed with the plunger top of the syringe through a 70 µm cell strainer. The cell strainer was rinsed with sterile cold PBS. Then the whole splenocyte suspension was centrifuged at 400 g for 5 min. The supernatant was discarded and the cell pellet was resuspended in normal saline at the concentration of 10^7 cells/100 µl. Cell solution was frozen at -80 °C, thawed at 37 °C for three cycles, followed by centrifugation at 17,000 g for 30 min at 4 °C. The supernatant (Spleen Soup) was pooled into a new tube and then was stored in a -80 °C freezer until injections into the recipient mice.

Adipose-derived stromal cells (ADSCs) were isolated and expanded in accordance with the established protocols[23]. In brief, inguinal adipose tissues were isolated from donor male

mice and minced into small pieces. After digestion with 0.075% collagenase type I for 30 min at 37°C, Adipose-derived stromal cells (ADSCs) was pelleted by centrifugation at 2000 g for 5 min and expanded in alpha-MEM supplemented with 15% FBS, 1% PFS, 1% L-Glutamine. Then, multi-lineage differentiation assays were performed as described previously to confirm the multipotency of these cells[22]. At passage 3, ADSCs were harvested with 0.25% trypsin-EDTA and washed with sterile PBS for 3 times. The cell pellet was resuspended in normal saline at the concentration of 2 x 10^6 cells/100 µl. Cell solution was frozen at -80 °C, thawed at 37 °C for three cycles, followed by centrifugation at 17,000 g for 30 min at 4 °C. The supernatant (ADSC Soup) was pooled into a new tube and then was stored in a -80 °C freezer until injections into the recipient mice.

Characterization of cell soups

The angiogenesis-related factors within BM Soup, Spleen Soup, and ADSC Soup were detected with Proteome Profile Mouse Array (ARY015, R&D Systems, Minneapolis, USA) as described previously[5]. In brief, 400 µg of total protein from cell soups was mixed with a cocktail of biotinylated detection antibodies and then incubated with proteome profile array membrane at 4°C overnight. Normal saline (vehicle) served as a negative control. After the unbounded antibodies were washed away, streptavidin-HRP and chemiluminescent detection reagents were applied subsequently. Lastly, membranes were exposed to X-Ray films to capture a signal. After subtracting the background and removing the overlaps with vehicle control, the signal corresponding to the amount of protein bound was analyzed by Image J software (NIH).

Irradiation (IR)

The animal model was induced by irradiation as described previously[5]. In brief, female C3H mice were anesthetized with 0.3 µl/g body weight of 60 mg/ml Ketamine and 8 mg/ml Xylazine (02239093, Novopharm, Toronto, Canada) and restrained in a container. Salivary glands were exposed to a single 13Gy of radiation from a Varian Clinac 6 EX linear accelerator (6 MV). High-energy photons were confined to mice SG area to spare the surrounding normal tissues and keep the out-of-field dose below 3% of the central axis dose[24]. The Mice were rehydrated with 500 µl normal saline given subcutaneously after irradiation. 100 µl vehicle (normal saline), BM Soup, Spleen Soup, or ADSC Soup was injected at 5~7 days post-IR, twice a week (every Monday and Thursday morning) for two consecutive weeks, into the tail vein (n=6). Mice were sacrificed at 8 & 16 weeks post-IR and their submandibular glands were harvested for further investigation.

Salivary flow rate (SFR)

SFR levels of the recipient mice were measured as previously described[25]. After anesthesia, Pilocarpine was injected at 0.5 mg/kg body weight to stimulate saliva secretion. The whole saliva was collected into a pre-weighed microcentrifuge tube for 10 min post-stimulation. SFR level was determined by volume of saliva/ min/ g body weight.

Periodic acid-Schiff stain (PAS)

Periodic acid-Schiff stain was performed to detect the functional acinar cells according to the manufacturer instructions (395B, Sigma-Aldrich, St-Louis, USA). Briefly, salivary gland tissues were fixed and embedded in paraffin. Five-micrometer thickness tissue sections were subsequently stained with periodic acid and Schiff's reagent. The percentage of surface area occupied by acinar cells was calculated by two independent examiners

under examiners under 200 x magnification of 10 fields per gland / mouse with Image J software (NIH).

Cell proliferation

PCNA staining was used to detect proliferating cells in SG tissue. Five-micrometer sections were blocked for endogenous peroxidase activity within 3% H_2O_2 for 10 min. Heat-induced antigen retrieval was conducted at 95°C for 10 min within 10mM citrate buffer (pH 6.1) and then cooled down to room temperature. Thereafter, slides were proceeded with IHC routine according to the manufacturer's instructions. The PCNA positive cell percentage was counted by two examiners in a blind manner under 400X magnification of 10 fields per gland/mouse with Image J software.

Immunofluorescent (IF) staining

Frozen tissue sections were fixed in 4% PFA for 10 min and incubated in 10 % normal donkey serum for 1 hour. Blood vessel endothelial cells were detected by Goat anti-CD31 antibody (1:200, AF3628, R&D Systems, Minneapolis, USA), while parasympathetic nerves were detected by Goat anti-GFRα2 antibody (1:200, AF429, R&D Systems, Minneapolis, USA). PBS was used as negative control. Frozen sections were incubated with primary antibodies or PBS at 4°C overnight and then with donkey anti-goat-fluorescein isothiocyanate-conjugated (FITC, 1:400). 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen, San Francisco, CA, USA) was applied to counterstain the nucleus of cells. Leica DM4000 fluorescent microscope and Volocity software were used to take pictures. The intensity of fluorescence signal was calculated with Image J software for 200 x of 5 fields / glands.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the submandibular glands with RNeasy Plus Mini Kit (74134, Qiagen, Valencia, CA91355, USA). First strand cDNA was synthesized from 25ng RNA per sample with High-Capacity cDNA Reverse Transcription Kit (4368814, ThermoFisher Scientific, MA02451, USA). QRT-PCR assays were conducted by Step One Plus (Life Technologies) in Taqman Universal. The probes used in this study were EGF (Assay ID: Mm00438696), VEGF (Assay ID: Mm01281449), IGF-1R (Assay ID: Mm00802841), HGF (Assay ID: Mm01135193), FGF2 (Assay ID: Mm01285715) and MMP9 (Assay ID: Mm01285715). GAPDH (Assay ID: Mm99999915) was used as the endogenous reference. Three experiment replicates were conducted for each sample.

Statistical analysis

SPSS version 19 was used to perform the statistical analysis. Student's *t*-test or one-way ANOVA analysis with Tukey's Post-Hoc test was conducted to compare the mouse groups. Statistical difference was defined as $p<0.05$.

Results:

Characterization of ADSCs.

The morphology of the ADSCs isolated in the study was fibroblast-like. To confirm the multipotency, multi-lineage differentiation assays were performed. ADSCs were able to undergo adipogenic, osteogenic, and chondrogenic differentiation when culturing in the specific induction medium, indicating that these cells were multipotent mesenchymal stromal cells （Fig. 1）.

Characterization of BM Soup, Spleen Soup, and ADSC Soup

Previously, we reported that proteins are the active ingredients in BM Soup and several angiogenesis-related factors and cytokines were identified[5]. To preliminarily compare the protein components of different cell soups, a protein microarray was used to profile the angiogenesis-related factors (Fig. 2a). After subtracting the background and removing the overlaps in normal saline, densitometric analysis was performed with Image J software. The results showed that Spleen Soup contained a significantly higher level of MMP-9, lower level of OPN and SDF-1, and comparable level of FGF-1, PF4, CD26, and MMP-8 to the BM Soup. However, no HGF was found in Spleen Soup (Fig. 2b). Interestingly, 18 proteins were found to be present in ADSC Soup, including pro-angiogenetic factors, such as TF, FGF-2, OPN, Ang-3, and MMP-3 et al., as well as the anti-angiogenetic factors, such as endostatin, IGFBP-3,-9, PTX3, and PAI-1 et al. (Fig. 2c). Since the antibody affinities for target antigens are different on the microarray membrane, it is unfeasible to quantitively compare the level of different proteins. The protein microarray results demonstrated that different cell soups contain different protein components. However, further investigation is required to identify the factors responsible for the therapeutic effect.

Cell soups from spleen and adipose tissues functionally restore IR-injured salivary glands

In the first part of this study, BM Soup, Spleen Soup, and ADSC Soup were injected into mice with IR-damaged SGs, respectively. Salivary flow rate (SFR) of the mice, as a critical measure of SG function, was compared in a dynamic way (Fig. 3a). As expected, 13 Gy of single dose irradiation resulted in a 50% reduction in SFR level at 1 week post-IR and this decreased further to 70% reduction when compared with the baseline of sham-IR mice during the 16 weeks follow-up period. Mice treated with BM Soup, Spleen Soup, or ADSC Soup had a comparable SFR level at 8 weeks post-IR, which was significantly higher than

that of the mice injected with the vehicle. During the longer follow-up period, this SFR levels were reduced to 50% of baseline at most in the BM Soup treated and Spleen Soup treated groups, while only 43.6% in the ADSC Soup treated group. In addition, at 16 weeks post-IR, BM Soup treated mice had a significantly higher SFR level than the mice treated with ADSC Soup.

Acinar cells are the principle site for fluid secretion and provide most of the secreted proteins in the saliva. The proportion of acinar cells is correlated with the secretion of saliva. In this study, functional acinar cells were detected with PAS staining at 8 weeks and 16 weeks post-IR (Fig. 3c&d). The results demonstrated that at both time points, the percentage of surface occupied by acinar cells / total area in all cell soup groups was higher than the saline injected group. Moreover, BM Soup treated mice had comparable acinar cell area to Spleen Soup group, but significantly higher than the ADSC Soup group. Cell proliferation rate was also measured with PCNA staining (Fig. 3b). Interestingly, ADSC Soup treated group had a comparable percentage of proliferating cells to the other two soup treated groups, which were all higher than the saline control group.

Blood vessel endothelial cells (CD31) and parasympathetic nerves (GFRα2) were detected with immunofluorescent staining. The results suggested a similar trend observed with acinar cell area. All cell soup treated groups had higher blood vessel density (Fig. 4a) and parasympathetic innervation (Fig. 4b) than the saline control group. However, ADSC Soup treated mice showed a slightly lower level than BM Soup and Spleen Soup group, although no significant difference was found. In accordance with the other measures, the expression levels of genes related to tissue repair/regeneration were upregulated in BM Soup and Spleen Soup group at 8 weeks post-IR, especially, Spleen Soup group had even higher

levels for HGF and MMP-9 genes than BM Soup group (Fig. 5). However, ADSC group did not show any significant difference with saline control group at this time point.

Cryopreserved BM Soup maintained the therapeutic effect against irradiation damage.

To test the influence of cryopreservation on the therapeutic effect of BM Soup, BM Soup was stored in -80 °C freezer for 12 months and then injected into mice with IR-damaged SG. The SFR level, acinar cell area, and cell proliferation rate were measured and compared with the freshly prepared BM Soup. As depicted in Figure 6, mice injected with cryopreserved BM Soup had comparable SFR level with the fresh BM Soup treated mice (Fig. 6a). Moreover, no significant difference was found in the acinar cell area (Fig. 6b) and cell proliferation rate (Fig. 6c) between cryopreserved and fresh BM Soup groups. All these results indicated that BM Soup could be stored at -80 °C freezer for up to 12 months without losing the activity.

Discussion

In this study, we found that 1) Spleen Soup has comparable therapeutic effects to the BM Soup on the restoration of SG hypofunction during 16 weeks follow-up period, while ADSC Soup had a relatively shorter effective period, although ADSC Soup contains more angiogenesis-related proteins. 2) BM Soup can be stored in -80 °C freezer for up to 12 months without losing the activity.

Three types of cell soup all originated from mice were tested in the mice with IR-injured SGs. Regarding the concentration of cell suspension, 10^7 cells/100 μl of BM Soup was used in the previous study and confirmed to be effective. In order to compare with BM Soup, we continued to use the same concentration for Spleen Soup. Moreover, preclinical

studies reported that 1 x 10^6 ADSC cells per injection were able to restore the function of IR-injured SGs[11]. In our preliminary study, the efficiency of cell lysis by freeze/thaw for ADSCs is around 50%. Therefore, 2 x 10^6 cells/100 µl was used to prepare ADSC Soup in this study. The total protein concentrations of BM Soup and Spleen Soup were 2.04 mg/ml and 2.27 mg/ml, respectively, while ADSC Soup was 4.24 mg/ml. To increase the efficiency of ADSC lysis, other strategies could have been used. For example, one cycle of freezing in liquid nitrogen for 5 min and thaw at 37 °C water-bath is enough to completely lysis the cells.

The major finding of this study was that Spleen Soup has a comparable effect to BM Soup and both can last longer time than ADSC Soup, although the latter had a higher total protein concentration. During the first 8 weeks follow-up, all three cell soups were found to comparably restore the saliva secretion. However, after that time, ADSC Soup lost its effect more rapidly than the other two soups. Both of Spleen Soup and BM Soup were from the whole cells, including all cell types of spleen and BM, while ADSC Soup was from relatively purified MSCs. These results are in agreement with studies on other organs. Angeli F.S. and colleagues compared the effects of whole BM Soup and mononuclear fraction Soup in a myocardium infarction mouse model and found that both cell soups resulted in a smaller infarct size post-MI, but whole BM Soup treated mice had more vessels in the border zone[6]. Then, we concluded that cell soup from whole cells harvested from an organ is superior to that from fractionated cells. Probably, it is unnecessary to purify the stem cells for cell soup treatment which would be easier and more feasible for the clinical application.

In addition, our group reported previously that repeated BM Soup injection resulted in an added beneficial improvement. We deduced that more frequency of ADSC Soup injections would maintain its therapeutic effect for longer time. Considering its advantages on harvesting and expansion, ADSC Soup could be a substitute for BM Soup. Although spleen is not easy to be obtained clinically as adipose tissue, our study provided a proof of concept that Spleen Soup has comparable effects to BM Soup. For some trauma cases, their spleens could be used for Spleen Soup preparation and cryopreserved for future clinical application, rather than discarded.

Protein microarray analysis demonstrated that BM Soup and Spleen Soup contain several overlapping angiogenesis-related factors, including MMP-8, -9, FGF-1, SDF-1, CD26, OPN, and PF4. Spleen Soup was missing HGF, but contained a higher level of MMP-9 when compared to BM Soup. HGF was involved in the branching morphogenesis of SGs[26], while MMP-9 was able to degrade the matrix surrounding the endothelial cells and promote their proliferation and migration[27]. Though BM Soup and Spleen Soup are slightly different regarding the angiogenesis-related protein components, both of them were found to protect blood vessel endothelial cells against the IR damage and result in a comparable blood vessel density detected by CD31 IF staining. ADSCs were isolated and purified from stromal vascular fraction (SVF), which consisted of a variety of cell population, such as endothelial cells, pericytes, and adipocyte progenitor/stem cells[23]. Thus, it is reasonable that more angiogenesis-related factors are present in cell extract from these cells. Interestingly, ADSC Soup was not superior to BM Soup and Spleen Soup on the blood vessel density of SGs. We hypothesize that it could be due to: 1) cell soup is cocktail which contains numerous cytokines, growth and other paracrine factors. Besides the

angiogenesis-related proteins detected in this study, other factors could also participate in the regeneration process and be responsible for the therapeutic effects. For example, Spleen Soup is from whole spleen cells which consisted of mainly lymphocytes and blood cells. There could be more cytokines than other two cell soups. BM Soup might contain more factors from mesenchymal or hematopoietic progenitor/stem cells. 2) Besides the pro-angiogenetic factors, ADCS Soup contained several anti-angiogenetic factors as well. Due to the limitation of microarray, it is unfeasible to compare ADSC Soup with other two soups on the proportion of pro- and anti-angiogenetic factors. However, relatively higher levels of anti-angiogenetic factors in ADSC Soup could be an alternative explanation.

Our previous study reported that the time-window of BM Soup treatment is limited to the early stage of IR damage. However, fresh cell soup is not always available in the clinic. Long-term storage of cell soups or cell soup banking may be important for clinical translation of cell soup treatment. Proteins are the main active ingredients in BM Soup, and thus several strategies could be used to preserve it, including -80 °C freezer, liquid nitrogen, and lyophilisation. However, liquid nitrogen or lyophilisation is not always available in normal clinic. In this study, we simply tested cryopreservation in -80 °C freezer for 12 months. The results demonstrated that this simple strategy did not affect its therapeutic effect, indicating that cell soup banking is achievable. It makes it possible to recycle some organs, such as a broken spleen.

Admittedly, there are limitations to this study. First, all these cell soups were from mice. It still remains unclear if the benefit can be translated to human cell soups. As human BM Soup has already been tested on myocardium infarction mouse model[6], we expected that this effect could be translatable to human cells. To confirm this hypothesis, our ongoing

experiment is using cell extract from human periodontal ligament stem cells (huPDL) to treat IR-induced salivary hypofunction in a mouse model. In addition, the frequency and concentration of cell soups were not optimized. The purpose of this study was to provide a proof of concept for the effect of different cell soups. Optimal treatment strategy needs further investigations. Lastly, BM Soup was only preserved for 12 months. In the future, longer preservation would be tested, but lyophilisation is recommended.

References:

1 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2016. *CA Cancer J Clin* **66**, 7-30 (2016).

2 Cotrim, A. P., Sowers, A., Mitchell, J. B. & Baum, B. J. Prevention of irradiation-induced salivary hypofunction by microvessel protection in mouse salivary glands. *Mol Ther* **15**, 2101-2106 (2007).

3 Vissink, A., Jansma, J., Spijkervet, F. K., Burlage, F. R. & Coppes, R. P. Oral sequelae of head and neck radiotherapy. *Crit Rev Oral Biol Med* **14**, 199-212 (2003).

4 Tran, S. D. *et al.* Paracrine effects of bone marrow soup restore organ function, regeneration, and repair in salivary glands damaged by irradiation. *PLoS One* **8**, e61632 (2013).

5 Fang, D. *et al.* Identification of the active components in Bone Marrow Soup: a mitigator against irradiation-injury to salivary glands. *Sci Rep* **5**, 16017 (2015).

6 Angeli, F. S. *et al.* Injection of human bone marrow and mononuclear cell extract into infarcted mouse hearts results in functional improvement. *Open Cardiovasc Med J* **6**, 38-43 (2012).

7 Stroncek, D. F. *et al.* Experiences of the first 493 unrelated marrow donors in the National Marrow Donor Program. *Blood* **81**, 1940-1946 (1993).

8 Buckner, C. D. *et al.* Marrow harvesting from normal donors. *Blood* **64**, 630-634 (1984).

9 Zuk, P. A. *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7**, 211-228 (2001).

10 Li, Z. *et al.* Protective efficacy of intravenous transplantation of adipose-derived stem cells for the prevention of radiation-induced salivary gland damage. *Arch Oral Biol* **60**, 1488-1496 (2015).

11 Lim, J. Y. *et al.* Systemic transplantation of human adipose tissue-derived mesenchymal stem cells for the regeneration of irradiation-induced salivary gland damage. *PLoS One* **8**, e71167 (2013).

12 Kojima, T. *et al.* Regeneration of radiation damaged salivary glands with adipose-derived stromal cells. *Laryngoscope* **121**, 1864-1869 (2011).

13 Kalinina, N. *et al.* Characterization of secretomes provides evidence for adipose-derived mesenchymal stromal cells subtypes. *Stem Cell Res Ther* **6**, 221 (2015).

14 Lee, S. M., Lee, S. C. & Kim, S. J. Contribution of human adipose tissue-derived stem cells and the secretome to the skin allograft survival in mice. *J Surg Res* **188**, 280-289 (2014).
15 Faustman, D. L. Regenerative medicine: Stem cell research turns to the spleen. *Discov Med* **5**, 447-449 (2005).
16 Faustman, D. L. & Davis, M. Stem cells in the spleen: therapeutic potential for Sjogren's syndrome, type I diabetes, and other disorders. *Int J Biochem Cell Biol* **42**, 1576-1579 (2010).
17 Dieguez-Acuna, F. *et al.* Proteomics identifies multipotent and low oncogenic risk stem cells of the spleen. *Int J Biochem Cell Biol* **42**, 1651-1660 (2010).
18 Robertson, S. A., Rowan-Hull, A. M. & Johnson, P. R. The spleen--a potential source of new islets for transplantation? *J Pediatr Surg* **43**, 274-278 (2008).
19 Lonyai, A., Kodama, S., Burger, D. & Faustman, D. L. Fetal Hox11 expression patterns predict defective target organs: a novel link between developmental biology and autoimmunity. *Immunol Cell Biol* **86**, 301-309 (2008).
20 Swirski, F. K. *et al.* Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* **325**, 612-616 (2009).
21 Tran, S. D. *et al.* Reversal of Sjogren's-like syndrome in non-obese diabetic mice. *Ann Rheum Dis* **66**, 812-814 (2007).
22 Khalili, S. *et al.* Bone marrow cells are a source of undifferentiated cells to prevent Sjogren's syndrome and to preserve salivary glands function in the non-obese diabetic mice. *Int J Biochem Cell Biol* **42**, 1893-1899 (2010).
23 Bunnell, B. A., Flaat, M., Gagliardi, C., Patel, B. & Ripoll, C. Adipose-derived stem cells: isolation, expansion and differentiation. *Methods* **45**, 115-120 (2008).
24 Papaconstadopoulos, P., Tessier, F. & Seuntjens, J. On the correction, perturbation and modification of small field detectors in relative dosimetry. *Phys Med Biol* **59**, 5937-5952 (2014).
25 Sumita, Y. *et al.* Bone marrow-derived cells rescue salivary gland function in mice with head and neck irradiation. *Int J Biochem Cell Biol* **43**, 80-87 (2011).
26 Ikari, T. *et al.* Involvement of hepatocyte growth factor in branching morphogenesis of murine salivary gland. *Dev Dyn* **228**, 173-184 (2003).
27 Bendeck, M. P. Macrophage matrix metalloproteinase-9 regulates angiogenesis in ischemic muscle. *Circ Res* **94**, 138-139 (2004).

Conflict of interest

The authors declare no competing financial interests.

Author Contributions

This study was designed by D.F., Y.L., J.S., and S.T.; D.F., Y.L., J.L., and J.S. conducted experiments. D.F., J.S., and S.T. wrote the manuscript. S.T. supervised this study and directed final version of all contents. All authors reviewed and approved the manuscript.

Acknowledgements

This study was supported in part by the Canadian Institutes of Health Research (CIHR) [Grant number: MOP-119585]. We thank Dr Mari Kaartinen for access to laboratory equipment. We also thank the Center for Bone and Periodontal Research (McGill University).

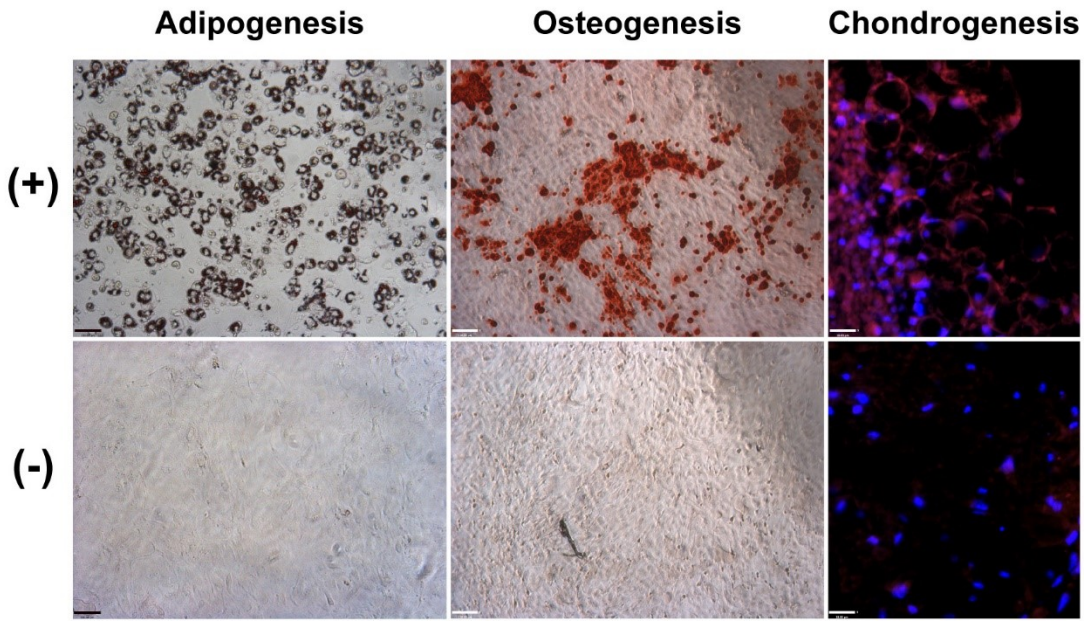

Figure 1. Characterization of adipose-derived stromal cells (ADSCs). ADSCs were induced to undergo adipogenic, osteogenic, and chondrogenic differentiation. Oil red staining and Alizarin Red staining were used to identify adipocytes and osteoblasts, respectively, while Collagen II for Chondrocytes.

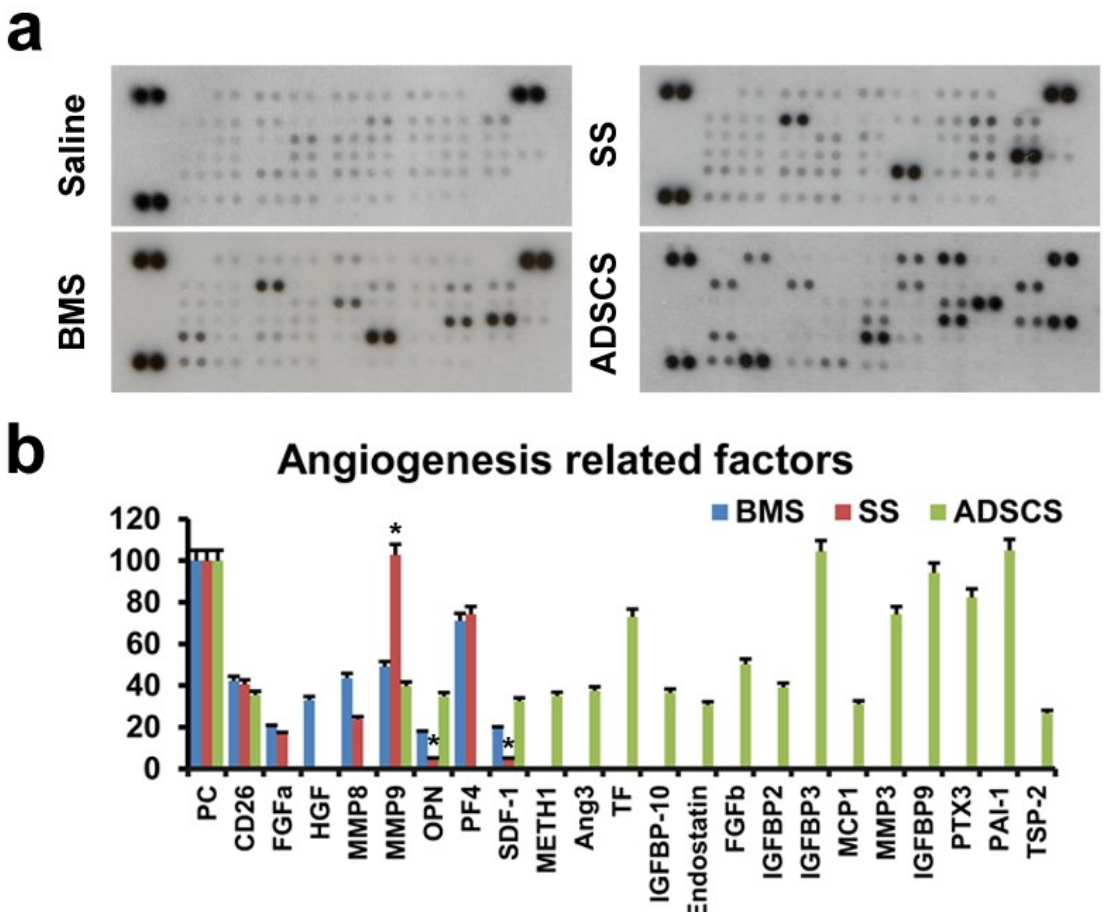

Figure 2. Screening of angiogenesis-related factors in Cell Soups originating from three different tissues. (a) A protein array was used to identify angiogenesis-related growth factors present in BM Soup, Spleen Soup, and ADSC Soup, while saline served as the negative vehicle control. (b) Semi-quantification of angiogenesis-related factors (intensity) present in each Cell Soup. All data were presented with mean ± S.D. and normalized to the positive control (three pairs of dots at the corners of the protein array). * means $p < 0.05$. ADSCS, adipose-derived stromal cell soup; BMS, bone marrow soup; SS, spleen cell soup.

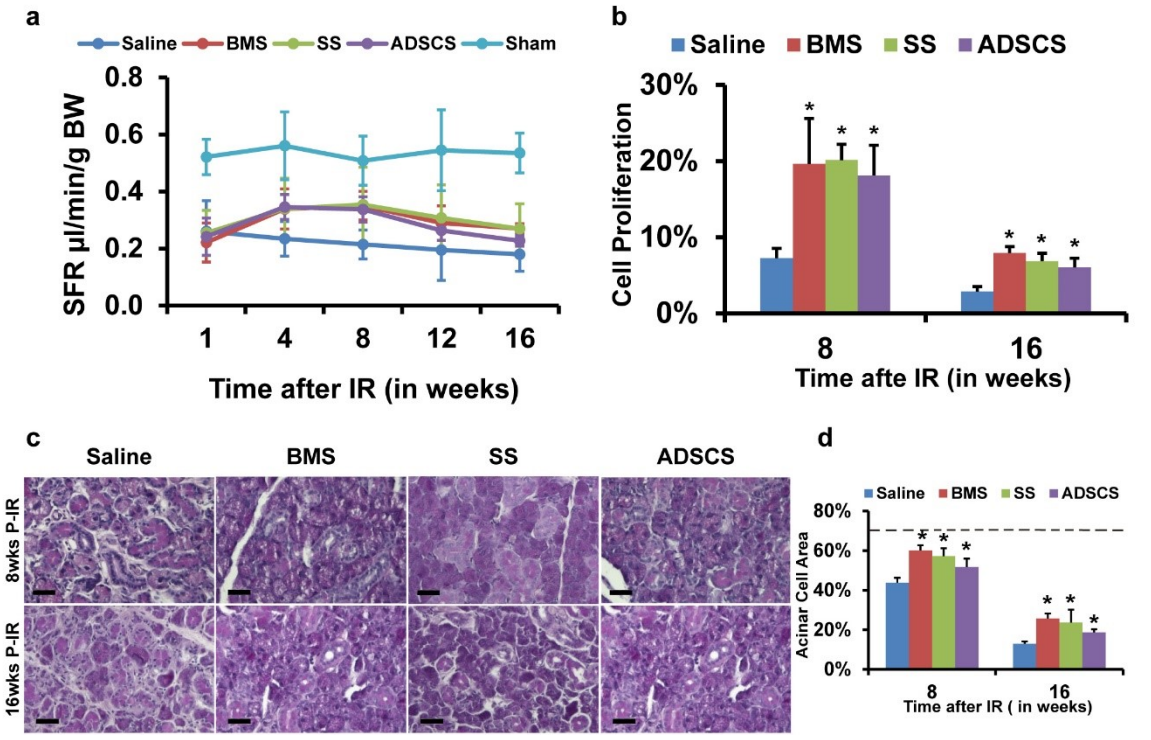

Figure 3. Effects of cell soups on salivary flow rate (SFR), acinar cells, and cell proliferation. At 5~7 days post-IR, BM Soup, Spleen Soup, or ADSC Soup was injected into the tail vein of mice with 13 Gy of irradiation to the head and neck area. Non-IR mice and irradiated mice with saline injection served as positive and negative control, respectively. **(a)** Salivary flow rate was measured at 1 week post-IR (before treatment), 4, 8, 12, and 16 weeks post-IR, and determined by µl/min/g body weight. The mice were sacrificed and their submandibular glands were harvested at 8 and 16 weeks post-IR. **(b)** Cell proliferation rate was calculated by counting PCNA positive cells under 400X magnification of 10 fields per gland/mouse with Image J software (NIH). **(c)** PAS staining was used to detect the functional acinar cells under 200X magnification of 10 fields per gland/mouse. Scale bar=38 µm. **(d)** The percentage of surface area occupied by acinar cells was shown. All data were presented with mean ± S.D. BMS: Bone Marrow Soup; SS: Spleen Soup; ADSCS: Adipose-derived Stromal Cell Soup.*$p<0.05$.

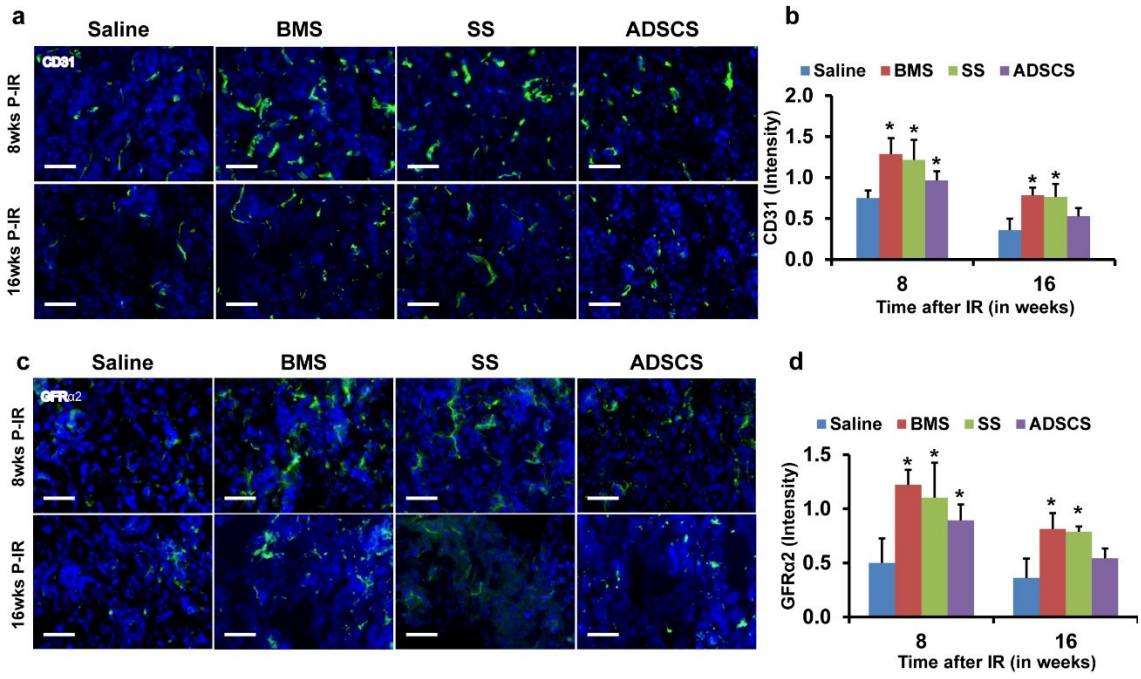

Figure 4. Blood vessels and parasympathetic nerves of salivary glands were detected by immunofluorescent staining. The cells positive for **(a)** CD31 (marker for blood vessel endothelial cell) and **(c)** GFRα2 (marker for parasympathetic nerve) were detected on frozen sections of salivary glands. Scale bar=38 μm. Semi-quantification of immunofluorescent expression for **(b)** CD31 and **(d)** GFRα2. All data were presented with mean ± S.D. BMS: Bone Marrow Soup; SS: Spleen Soup; ADSCS: Adipose-derived Stromal Cell Soup. *p<0.05

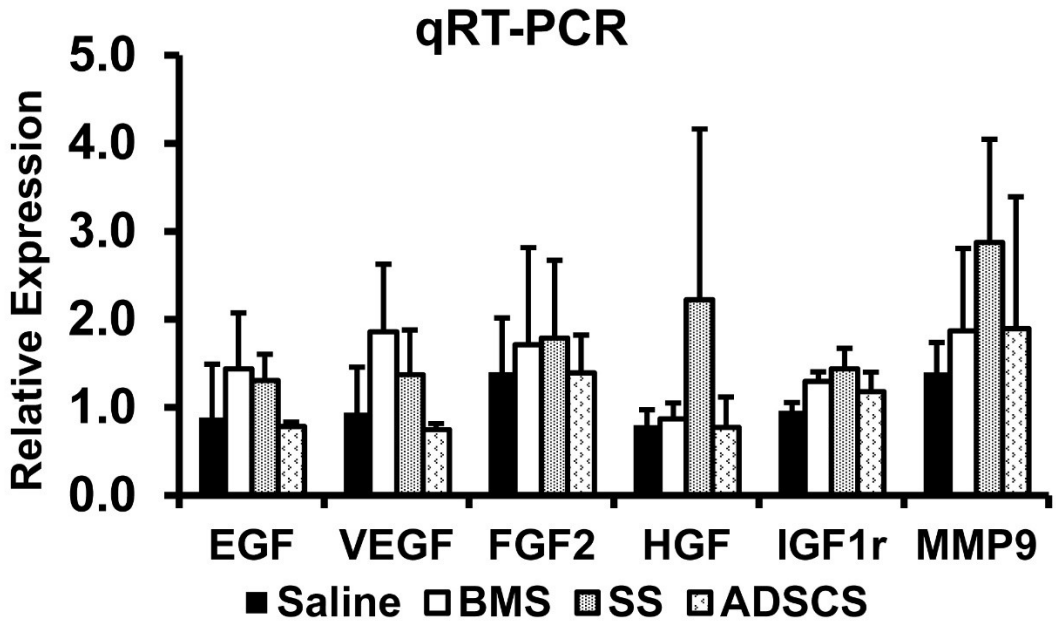

Figure 5. Expression of genes in salivary glands at 8 weeks post-IR. The relative expression of genes related to tissue repair/regeneration was determined by qRT-PCR. All genes tested in this study were upregulated in both BM Soup and Spleen Soup treated group. GAPDH was used as the endogenous reference. Three experiment replicates were performed for each sample. All data were presented with mean ± S.D. BMS: Bone Marrow Soup; SS: Spleen Soup; ADSCS: Adipose-derived Stromal Cell Soup.

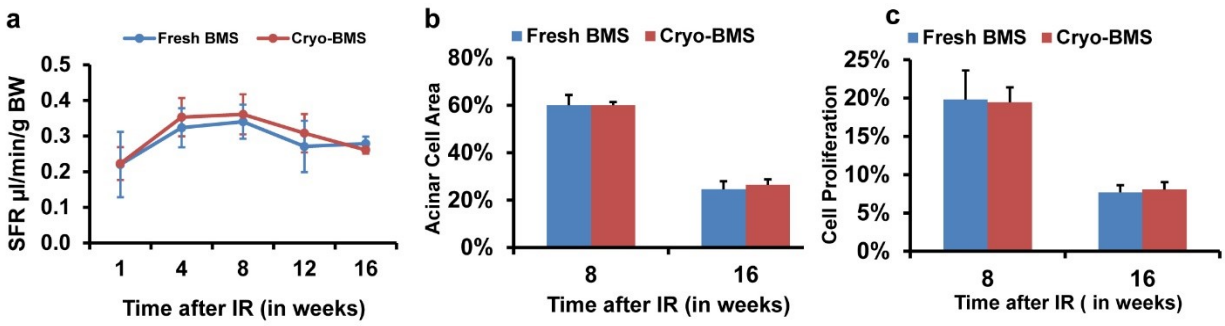

Figure 6. Comparison of fresh BM Soup and cryopreserved BM Soup. Both fresh BM Soup and BM Soup cryopreserved for 12 months (Cryo-BMS) were injected into mice with IR-injured SGs. **(a)** Salivary flow rate was measured at 1 week post-IR (before treatment), 4, 8, 12, and 16 weeks post-IR, and determined by µl/min/g body weight. The mice were sacrificed and their submandibular glands were harvested at 8 and 16 weeks post-IR. **(b)** The percentage of surface area occupied by acinar cells was calculated under 200X magnification of 10 fields per gland/mouse with Image J software (NIH). **(c)** Cell proliferation rate was calculated by counting PCNA positive cells under 400X magnification of 10 fields per gland/mouse. All data were presented with mean ± S.D.

CONCLUSION AND FUTURE DIRECTION

Conclusion:

Salivary hypofunction is a major side effect of radiotherapy for patients with head and neck cancer. However, current treatments remain palliative, and no restorative therapy is available in the clinic. Our group reported that cell extract from bone marrow, named 'BM Soup', alleviated the IR damage to the salivary glands. BM Soup includes all cell types of whole BM and consequently numerous proteins, cytokines, paracrine factors. Because BM Soup contains the cell by-products, it does not require the injection/ transplantation of whole live cells which carry the risk of differentiating into tumorigenic cell types. It does not need to be grown in culture or harvested with a cell-derived conditioned media, which is time-consuming and carries the risk of genetic modification. BM Soup is easier to store/ preserve than BM cells, and it can be easily adapted to existing transfusion medicine protocols and used in a clinical setting. However, the bioactive ingredients of BM Soup which are responsible for the therapeutic effects still remain unknown. In Chapter 2 of this thesis, we demonstrated that protein components, but not the nucleic acids, lipids, or carbohydrates, are the therapeutic ingredients in BM Soup for functional restoration of IR-injured SGs. Moreover, several angiogenesis-related factors are found, such as CD26, FGF, HGF, MMP-8, MMP-9, OPN, PF4, and SDF-1.

For any given therapy, the frequency and timing of treatment are the important parameters in the restoration of salivary glands. The study presented in Chapter 3 optimized the frequency and timing of BM Soup injection. Starting injections of BM Soup within 3 weeks post-radiation maintain 90-100 % of saliva flow in radiated mice, while delayed treatment had no saliva secretion improvement. In addition, increased frequency of BM Soup injections results in an added beneficial improvement.

Although BM Soup can mitigate the IR injury to the SGs, BM cell harvesting remains an invasive procedure and could lead to some complications. Chapter 4 tested other types of cell soup and showed that adipose-derived cell soup and spleen soup had comparable therapeutic effects with BM Soup at eight weeks post-IR, but BM Soup and Spleen Soup maintain the effects for a longer time.

This thesis preliminarily investigated the mechanism of BM Soup treatment, optimized the frequency and timing of injections, and tested the effects of other cell soups. It provides the experimental basis for the future clinical application of this cell-free molecular therapy.

Future plan:

- Investigating the underlying molecular mechanisms of BM Soup treatment. Both RNA-Seq and quantitative proteomics will be used to identify genes and associated pathways in SG targeted by BM Soup treatment. Once these genes and proteins are identified, the effective molecular components in BM Soup for functional restoration of damaged SGs in IR-injured mice will be validated by using a combination of immune-depletion, proteome array profiling, and animal models.
- Assessing the safety of BM Soup. In vitro and in vivo experiments on the safety of BM Soup will be performed on cell lines and healthy C3H mice, respectively. Moreover, considering BM Soup contains numerous growth factors, its effect on tumor growth will be tested on C3H mouse model for cancer.