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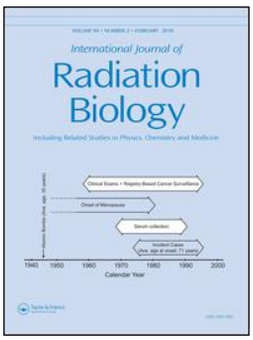

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Vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), angiostatin, and endostatin are increased in radiotherapy-induced gastrointestinal toxicity

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Running title: MMP inhibition and irradiation of tumour endothelial cells

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Abstract

Purpose: Radiotherapy-induced gut toxicity (RIGT) is a debilitating effect of radiotherapy for cancer, often resulting in significant diarrhoea and pain. Previous studies have highlighted roles of the intestinal microvasculature and matrix metalloproteinases (MMPs) in the development of RIGT. We hypothesized vascular mediators would be significantly altered in a dark agouti (DA) rat model of RIGT. Additionally, we aimed to assess the effect of MMP-2 and -9 inhibition on the response of tumour-associated microvascular endothelial cells (TAMECs) to radiation.

Methods: Dark Agouti (DA) rats were administered 2.5 Gy abdominal irradiation (3 times/week over 6 weeks). Vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), von Willebrand factor (VWF), angiostatin, and endostatin expression was assessed at 3, 6 and 15 weeks. Additionally, DA rat mammary adenocarcinoma tumour-associated microvascular endothelial cells (TAMECs) were used to assess the effects of radiation (12 Gy) and the MMP inhibitor SB-3CT on MMP, VEGF, and TGFβ expression, and cell viability.

Results: VEGF mRNA expression was significantly increased in the colon at week 15 ($p = 0.0012$), and TGFβ mRNA expression was significantly increased in both the jejunum and colon at week 3 ($p = 0.0280$, and $p = 0.0310$, respectively). Endostatin immunostaining was significantly increased at week 3 ($p = 0.0046$), and angiostatin at 3 and 6 weeks ($p = 0.0022$, and $p = 0.0135$, respectively). MMP-2 and -9 mRNA and total protein levels were significantly increased following irradiation of TAMECs. Although this increase was significantly attenuated by SB-3CT, it did not significantly alter endothelial cell viability or VEGF and TGFβ mRNA expression.

Conclusions: Findings of this study support the involvement of VEGF, TGFβ, angiostatin, endostatin, and MMP-2 in the pathobiology of RIGT. However, the relationship between these mediators is complex and needs further investigation to improve understanding of their therapeutic potential in RIGT.

Introduction

Radiotherapy-induced gut toxicity (RIGT), estimated to affect 60-80% of patients receiving radiotherapy for abdominal or pelvic tumours, induces debilitating symptoms including, but not limited to, diarrhoea, pain, rectal bleeding, and incontinence (Theis et al. 2010; Shadad et al. 2013; Hauer-Jensen et al. 2014). RIGT is often divided into acute and chronic toxicity, with acute changes involving histopathological damage to villi and crypts, and chronic involving reactive fibrotic thickening (Yeoh et al. 2007; Theis et al. 2010). Previous research has suggested the involvement of the intestinal microvasculature in the development of RIGT, and several mediators have been investigated (Kruse et al. 2004; Yeoh et al. 2007; Stansborough et al. 2017). NFκB, proinflammatory cytokines, and matrix metalloproteinases (MMPs) are all upregulated in the intestine following radiation, and have downstream effects on vascular mediators such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), von Willebrand factor (VWF), and the antiangiogenic mediators endostatin and angiostatin (Stansborough et al. 2016). Whilst a link has been proposed in RIGT (Stansborough et al. 2016) there is limited research which has investigated changes in these mediators in the intestinal microvasculature during a fractionated radiation schedule.

Our previous studies have shown the intestinal microvasculature to be involved in RIGT, with apoptosis, cytostasis, telangiectasis, and fibrotic thickening occurring early and late following fractionated irradiation in dark agouti (DA) rats (Yeoh et al. 2005; Stansborough et al. 2017). In addition, several regulators of angiogenesis and vascular physiology including, but not limited to, TGFβ, VEGF, and VWF, are known to be altered in the intestine following irradiation (Wang et al. 2001; Wang et al. 2002; Milliat et al. 2006; Lenting et al. 2012; Boerma et al. 2013). VEGF, bound to heparin in the extracellular matrix, is able to be released by matrix metalloproteinases (MMPs) such as MMP-2, and MMP-9 (Carmeliet 2005). The anti-angiogenic mediators angiostatin and endostatin have also been shown to increase in response to irradiation of cancer endothelial cells derived from human breast cancer tissue (Oh et al. 2014). A reduction in angiostatin via knockdown of plasminogen restored tube

formation of these cells, suggesting angiostatin is involved in endothelial regulation following irradiation (Oh et al. 2014). Although it appears angiostatin and endostatin are involved in endothelial response to irradiation this connection is yet to be explored in RIGT. With the increasing investigation of the possible use of anti-angiogenic agents as adjuvant cancer therapies alongside chemotherapy or radiotherapy, targeting mediators such as vascular endothelial growth factor (VEGF) and endostatin, the role of these mediators in RIGT needs to be further explored.

Previous research has clearly shown MMPs are involved in RIGT, with MMP-2 in particular shown to be significantly increased during RIGT (Strup-Perrot et al. 2006). It has also been hypothesised MMPs are involved in microvascular changes and alteration to vascular mediators such as VEGF, TGFβ, and VWF in RIGT (Stansborough et al. 2016). MMPs are able to regulate the expression of several of these mediators via the degradation of the extracellular matrix (ECM) leading to the release of latent VEGF and TGFβ, as well as forming angiostatin and endostatin by cleaving plasminogen and collagen XVIII respectively (Sternlicht and Werb 2001; Kalluri 2003; Carmeliet 2005). In degrading the ECM, MMPs also promote endothelial migration, proliferation and tube formation (Kalluri 2003). Despite previous literature, a direct link has not been established between MMPs and vascular mediator expression following radiation, and the effect of these changes on endothelial cells. Thus the present study aimed to first confirm the alteration of vascular mediators VEGF, TGFβ, VWF, angiostatin, and endostatin in the jejunum and colon following fractionated irradiation in DA rats. We then aimed to determine whether inhibition of MMP-2 and -9 with SB-3CT altered endothelial viability and expression of these vascular mediators in primary tumour-associated microvascular endothelial cells (TAMECs) derived from DA rat mammary adenocarcinomas. TAMECs have been successfully cultured for previous, similar experiments, have been phenotyped, and their cultivation in DA rats allows for consistency between both components of the study (Bateman et al. 2013). In utilising this primary *in vitro* model we aimed to assess the effects of SB-3CT on tumour endothelial response to irradiation as an initial investigation into the response of tumour endothelium to MMP-2 and -9 inhibition. As the cell type used in the *in vitro*

component of the study does not directly translate to RIGT, further studies are required to assess the effects of SB-3CT on intestinal endothelium following irradiation. However, results of the present study form a foundation for further investigation into the possible use of SB-3CT following irradiation.

Materials and Methods

Ethics

Both studies were conducted in accordance with ethics approved by the Animal Ethics Committees at the University of Adelaide (M-041-2006; M-2015-117) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2013).

Animals and Experimental Design

Archival tissue from a prior study was utilised in the *in vivo* component of the present study, and experimental design has been previously described (Yeoh et al. 2007; Stansborough et al. 2017). Briefly, 30 female Dark Agouti (DA) rats were randomly assigned into two groups, one group receiving sham irradiation, and one group receiving a schedule of fractionated irradiation. Both groups of rats were anaesthetised using 3% halothane in 100% oxygen and placed into a custom-built container, with rats in the treatment group receiving fractionated abdominal irradiation using a Varian Clinac Linear Accelerator (Varian Medical Systems, USA) at a dose of 2.5 Gray (Gy) (depth = 3.3 cm, focus-skin distance = 130 cm) 3x per week for a total dose of 22.5 Gy in 9 fractions and 45 Gy in 18 fractions over 3 and 6 weeks, respectively (Yeoh et al. 2007). Rats were killed at 3, 6, or 15 weeks from first irradiation dose (n = 5 per time point). Animals were monitored daily and clinical record sheets kept. Sections of jejunum (30% of small intestinal length when measured from the pylorus) and colon (midlength of the large intestine) were collected as previously described and either formalin-fixed and paraffin-embedded, or snap frozen and stored at -80º (Yeoh et al. 2007; Stansborough et al. 2017).

RNA isolation and reverse transcription

RNA isolation was performed using the Nucleospin® RNA II kit (Macherey-Nagel, Germany) according to manufacturers' protocol. Briefly, 15-20 mg of jejunal and colonic tissue was homogenized using the TissueLyser LT (Qiagen) and Nucleospin® RNA II kit reagents (Macherey-Nagel, Germany). The lysate was filtered through silica membrane filter columns, desalted, DNA was digested using the DNAse reaction mixture, and the silica membrane was washed and dried. RNA was eluted in 60 µl RNase-free water. The TAKE3 plate and Synergy MyReader (BioTek) was used to determine RNA concentration and purity. RNA (1 µg) was converted to cDNA using the iScript cDNA synthesis (Bio-Rad, USA) according to manufacturer's protocol. The TAKE3 plate and Synergy MyReader was again used to determine total cDNA concentration and purity and stock cDNA was diluted to 100 ng/µl with nuclease-free water.

Real-time polymerase chain reaction (RT-PCR)

RT-PCR was performed using the Rotor Gene 3000 (Corbett Research, Sydney, Australia) according to manufacturer's protocol. Reaction mixtures contained 1 µl of cDNA, 5 µl of SYBR green (Applied Biosystems, Foster City, CA), 5 µl of nuclease-free water, and 0.5 µl each of 50 pmol/µl forward and reverse primers (Table 1). Thermal cycling conditions were as described; denaturation step at 95°C for 10 minutes, and included 40 cycles at 95°C for 10 s, annealing at 60ºC (VWF, TGFβ, and MMP-2), or 55ºC (VEGF and MMP-9), for 15 s, and extension and acquiring at 72°C for 20 seconds. 18S was used as the housekeeping gene. Primer efficiency was calculated to be between 90-110% for each target gene against 18S, and thus the $2^{-\Delta\Delta CT}$ method was used to determine fold change in gene expression relative to the housekeeping gene and sham irradiated controls from each time point.

Immunohistochemistry (IHC)

Angiostatin and endostatin proteins were visualised by IHC staining in the colon at 3, 6, and 15 week time points. A Dako automated staining system (Autostainer link 48 and PT Link, Dako, Denmark) was

used with the EnVision™ FLEX kit for Dako autostainer link systems (Dako, Denmark) as previously described (Stansborough et al. 2017). The anti-angiostatin primary antibody (#ab2904, Abcam), and anti-endostatin primary antibody (#GTX37706, GeneTex), were used at 6.66 µl/ml and 20 µl/ml, respectively. Slides were scanned using the Nanozoomer (Hamamatsu Photonics, Japan) at 40x magnification. ImageScope imaging software (Leica Biosystems, Germany) was used to analyse angiostatin and endostatin in which percentage of positively stained cells was calculated in the lamina propria of each section (averaged from ten randomly selected 40x sections per slide).

Isolation of tumour-associated microvascular endothelial cells (TAMECs)

Tumour-associated microvascular endothelial cells were isolated and cultured as previously described (Bateman et al. 2013). Briefly, Dark Agouti (DA) rats were injected subcutaneously with DA mammary adenocarcinoma cells, and 8 days following, tumours were removed and processed under sterile conditions. Surrounding connective tissue was removed and tissue was mechanically disrupted into 1 mm fragments. The tissue was enzymatically digested in collagenase/dispase (Sigma), and incubated with 0.25% trypsin/EDTA (Bateman et al. 2013). Following this, the tissue was centrifuged and resuspended in complete growth medium, consisting of supplemented Dulbecco's Modified Eagle Media (DMEM) (28% HEPES buffer, 10% foetal bovine serum (FBS), and 1% L-glutamine, penicillin, streptomycin) and filtered through a 100 µm nylon cell strainer (Corning). The resultant suspension was added to gelatinised culture flasks and incubated at 37ºC. Tumour-conditioned medium (TCM) was added to the TAMECs every 24-48 hours to maintain the endothelial cell culture. Endothelial culture was confirmed using immunostaining for endothelial cell markers, as previously described (Bateman et al. 2013).

Table 1. 18S, VEGF, TGFβ, VWF, MMP-2, and MMP-9 primer sequences

Treatment of TAMECs

TAMECs at passages 1-2 were detached with trypsin, pelleted, counted, diluted to 1 x 10^9 cells/mL, and split evenly across 4 x 50 ml falcon tubes to represent the four treatment groups (vehicle control, SB-3CT alone, irradiation alone, and irradiation and SB-3CT combined). Cells were then treated for 24 hours with either 0.01% DMSO in H20 (vehicle control), or 1 µm SB-3CT. SB-3CT concentration was determined by dose response assays (data not shown) and was determined to be within the K_i range of inhibiting MMP-2 and MMP-9 (K_i = 14 nM and K_i = 600 nM, respectively), without inhibiting MMP-1, -3, or -7 (K_i = 206 µM, K_i = 15 µM, K_i = 96 µM, respectively) (Brown et al. 2000). Following this, falcon tubes containing the cells were either sham irradiated, or received 12 Gy gamma irradiation using a Cs^{137} source (IBL-437 Blood Irradiator). Cells were immediately plated according to individual experimental conditions. An XTT assay was used to conduct a radiation dose response assay and was based on an initial dose range of 0-12 Gy. 12 Gy resulted in a significant decrease in cell viability without causing excessive cell death (data not shown). Dosimetry was calculated prior to radiation and based on dose rate calculations, calibrated monthly based on degradation constant.

Protein and RNA extraction from TAMECs

Following irradiation and/or SB-3CT pre-treatment, TAMECs were seeded into 6-well plates at a cell density of 1 x 10^5 cells/well, and incubated for 24 hours. Following incubation, the media was collected and immediately placed on ice. Cells were pelleted and supernatant was collected and stored at -80ºC. Lysis buffer (Macherey-Nagel RNA kit) was added to each well. Wells were scraped and cells and buffer were collected and disrupted using a 26-gauge needle. β-mercaptoethanol (3.5 µl) was added to each tube, vortexed, and further RNA extraction and cDNA conversion was performed according to kit protocols, as described earlier.

MMP 2 and 9 activity assays

MMP-2 and -9 activity was assessed in supernatant collected from treatment TAMECs, using MMP-2 and -9 activity assay kits (QuickZyme). Experiments were conducted according to kit protocol. Briefly, duplicate diluted samples (1:2 dilution with assay buffer), serially diluted standards, and assay buffer controls, were pipetted into the assay plate. The plate was covered and incubated at 4ºC overnight. The wells were aspirated and washed with assay buffer, and 50 µl of P-Aminophenyl mercuric acetate (APMA) solution (0.5 mM APMA in 0.05% DMSO/assay buffer) was added to wells containing standards, and to one of the two duplicate sample wells. Assay buffer was added to the remaining sample wells, and detection reagent was added to all wells. The plate was shaken for 20 seconds and read at 405 nm to obtain the baseline value, repeated at 6 and 22 hours. Data was analysed by subtracting the average blank value from each sample value, creating a standard curve based on the 6 hour data and plotting the standard concentration against the blank subtracted standard values, applying a best-fit curve, and calculating the sample concentrations graphically.

XTT Assay

In preparation for XTT assay, $1x10^5$ treated TAMECs were passaged intro 96-well flasks and were left to adhere for 24 hours. Following adhering, XTT was performed according to kit protocol (Cell Proliferation Kit II (XTT, Sigma), with labelling and electron coupling reagents being prepared immediately before removing media from wells, replacing with 100 µl fresh media, and adding 50 µl per well of the prepared XTT reagent. The plates were incubated for 6 hours and the plate was read at 490 nm using the TAKE3 plate and Synergy MyReader.

Immunocytochemistry (ICC)

Prior to experimentation of TAMECs, expression of VEGF, VWF, TGFβ, MMP-2, and MMP-9 under normal conditions was confirmed using immunocytochemistry (Supplementary Figure 1). Briefly, treated TAMECs were passaged into 8-well chamber slides at $2x10^5$ cells/well and left to adhere for 24 hours. Following this, cells were fixed with 4% paraformaldehyde for 30 minutes at 4ºC, washed twice with

PBS, incubated with 3% H2O2 in PBS for 5 minutes to reduce non-specific staining, washed, and incubated with Triton-X block (100 µl TX-100 and 0.1 g sodium citrate in 100 mL H20) for 8 minutes to permeate the cell membrane. The cells were washed and blocked using the normal serum blocking reagent (Level 2 USA™ Ultra Streptavidin Detection System kit, Signet Laboratories). Primary antibodies (MMP-2; #ab37150, Abcam, MMP-9; #ab58803, Abcam, VEGF; #ab46154, Abcam, VWF; #ab6994, Abcam, TGFβ; #ab92486, Abcam) were prepared at previously optimised concentrations of 2 µl/ml (VWF & MMP-2), 4 µl/ml (VEGF & MMP-9), and 10 µl/ml (TGFβ), and added to each well for 60 minutes. Cells were washed and a linking reagent (Level 2 USA™ Ultra Streptavidin Detection System kit, Signet Laboratories) was applied for 20 minutes. A peroxidase-labelled ultra-streptavidin labelling reagent (Signet Laboratories) was applied for 20 minutes, followed by a 1 minute incubation with diaminobenzidine (DAB) chromogen in 0.03% hydrogen peroxidase (Signet Laboratories) for visualization. Cells were washed, chambers were removed, and slides were counterstained with Lillie Meyers haematoxylin (1:10), washed, dehydrated, and coverslipped.

Statistical Analysis

Graphpad Prism (version 6) software was used to perform statistical analysis. Normality was determined and differences between study group means were analysed using either a one-way or two-way ANOVA with Sidak's multiple comparison, or a Kruskal-Wallis test with multiple comparison. Asterisks denote significance compared to control at the time point, where * <0.05, ** <0.01, *** <0.001, and **** <0.0001. Bars on graphs represent standard error of the mean.

Results

Fractionated irradiation induced changes in weight gain and histopathological damage

Clinical and histological response to fractionated irradiation for this study has been previously published (Stansborough et al. 2017). Briefly, rats receiving fractionated irradiation had significantly lower weight gain at week 3 ($p = 0.0055$), and from week 6 until the completion of the study ($p < 0.05$), when compared to sham irradiated controls (Stansborough et al. 2017). Histopathological damage scoring was significantly increased in the colon, but not in the jejunum, at week 3 ($p = 0.043$). However, thickening of the lamina propria, telangiectatic vessel formation, and apoptosis were observed in both the jejunum and colon following irradiation (Stansborough et al. 2017).

VEGF and TGFβ were significantly increased in the colon following irradiation in DA rats

Despite no significance effect in the jejunum, VEGF mRNA expression was significantly increased in the colon at the 15-week time point ($p = 0.0012$) (Figure 1A + B). TGFβ mRNA expression significantly increased in both the jejunum and colon 3 weeks into the radiation schedule ($p = 0.0280$ and $p = 0.0310$, respectively), however was not significantly increased at 6- or 15-weeks in either region (Figure 1C + D). VWF mRNA expression was not significantly altered at any time point following fractionated irradiation in jejunum or colon (Figure 1E + 1F).

Figure 1. VEGF, TGFβ, and VWF expression in the jejunum and colon following fractionated radiation. **A** VEGF mRNA expression in the jejunum following fractionated irradiation **B** VEGF mRNA expression in the colon following fractionated irradiation **C** TGFβ mRNA expression in the jejunum fractionated irradiation. **D** TGFβ expression in the colon following fractionated irradiation. **E** Relative mRNA expression of VWF in the jejunum following fractionated irradiation **F** VWF mRNA expression in the

colon. Dotted line represents baseline fold change. * = significance compared to control. * <0.05, ** <0.01, n=5. Data = mean + standard error of the mean (SEM).

Angiostatin and endostatin immunostaining was significantly increased in the colon following fractionated irradiation

Endostatin was immunostaining significantly increased in the lamina propria of the colon at the 3-week time point only (p = 0.0046) (Figure 2A + 2C). Angiostatin immunostaining was significantly increased at 3- and 6-weeks in the colon lamina propria (p = 0.0022, and p = 0.0135, respectively) (Figure 2B + 2C). There were no significant alterations at week 6 or 15.

Figure 2. *Endostatin (**A**) and angiostatin (**B**) immunostaining in the lamina propria of the colon following fractionated irradiation.* Cell positivity was calculated using ImageScope software and is determined as number of positively stained cells/number of total cells. * denotes significance compared to controls at the same time-points, ** <0.01, *** <0.001. Data = mean + SEM, n=5. Scale bars = 20 μm, 40x original magnification.

SB-3CT significantly attenuated the expression of MMP-9 following irradiation of TAMECs

Relative mRNA expression of MMP-2 was not significantly altered following irradiation of TAMECs when compared to controls (p = ns; Figure 3A). However, TAMEC MMP-2 expression following irradiation was significantly reduced when pre-treated with SB-3CT, as compared to the radiation only group (p = 0.0034) (Figure 3A). MMP-9 mRNA expression was significantly increased following irradiation of TAMECs (0.0022). This was significantly attenuated by SB-3CT (p < 0.0001). Despite this attenuation of MMP-2 and -9, SB-3CT did not significantly alter cell viability following irradiation, compared to radiation alone (p = ns; figure 5).

Figure 3. *MMP-2 (**A**) and -9 (**B**) mRNA expression following irradiation of TAMECs, with/without SB-3CT*. Dotted line represents baseline fold change. * = significance compared to control. * <0.05, ** <0.01, **** <0.0001. Data = mean + SEM. n=12

SB-3CT significantly attenuated MMP-2 and -9 protein expression following irradiation

Total protein levels of both MMP-2 and MMP-9 were significantly increased in TAMEC supernatant following 12 Gy irradiation ($p = 0.046$, and $p = 0.0401$, respectively) (Figure 4A and 4C). This increase in total MMP-2 and -9 protein levels was significantly attenuated by the administration of SB-3CT ($p = 0.0119$, and $p = 0.0209$, respectively). The ratio of active: total MMP-2 and -9 were not significantly altered following irradiation of TAMECs. However the ratio of active: total MMP-2 was significantly increased in the radiation + SB-3CT group, compared to control ($p = 0.0379$). The ratio of active: total MMP-9 was not significantly altered in the radiation + SB-3CT group when compared to all other groups (p = ns; Figure 4D).

Figure 4. *Total and active: total ratio of secreted MMP-2 and -9 protein concentration following irradiation and MMP inhibition in TAMECs.* **A** Total MMP-2 protein concentration **B** Ratio of active: total MMP-2 protein levels **C** Total MMP-9 protein concentration **D** Ratio of active: total MMP-9 protein levels. * = significance compared to control. * <0.05. Data = mean + SEM. n = 12

SB-3CT did not alter tumour-associated microvascular endothelial cell viability following irradiation

SB-3CT alone did not significantly alter cell viability; as measured via XTT assay; both irradiation (12 Gy), and irradiation + SB-3CT significantly reduced cell viability of TAMECs ($p = 0.0236$, and $p = 0.0003$, respectively) (Figure 5). There was no significant difference between the radiation alone, and radiation + SB-3CT groups (p = ns; Figure 5).

Figure 5. *Cell viability of TAMECs following irradiation and MMP inhibition.* * = significance compared to control. * <0.05, *** <0.001. Data = mean + SEM. *n = 8*

VEGF and TGFβ mRNA expression were not significantly altered following irradiation of TAMECs

RT-PCR was used to assess relative mRNA expression of VEGF and TGFβ following irradiation and MMP inhibition in TAMECs. VEGF and TGFβ mRNA expression were unaltered by both irradiation and SB-3CT treatment (Figure 6).

Figure 6. *VEGF and TGFβ mRNA expression following irradiation of TAMECs with or without SB-3CT.* VEGF (**A**) and TGFβ (**B**) mRNA expression were not significantly altered following irradiation of TAMECs, with or without the presence of SB-3CT. Dotted line represents baseline fold change. Data = mean + SEM. n = 12

Discussion

Although the pathobiology of RIGT is complex, MMPs, the intestinal microvasculature, and associated vascular mediators have been shown to be involved (Stansborough et al. 2016). Key findings from this study show mRNA expression of VEGF and TGFβ mRNA expression to be significantly increased in the intestines following RIGT, and is the first study to show an increase in angiostatin and endostatin during RIGT. However, the pattern of upregulation of these mediators was different, occurring at different time points and in different intestinal regions. Further, this study assessed the effect of the MMP-2 and -9 inhibitor, SB-3CT, on tumour-associated microvascular endothelial cells (TAMECs) following irradiation. Both mRNA and protein expression of MMP-2 and -9 were significantly upregulated in TAMECs following irradiation, however irradiation did not significantly alter VEGF or TGFβ expression in these cells. SB-3CTsignificantly attenuated MMP-2 and -9 mRNA and protein expression, however, it did not alter cell viability of TAMECs following irradiation.

VEGF mRNA expression was significantly increased in the colon at the 15 week time point of the DA rat RIGT model. VEGF may have a protective role in gastrointestinal injury, with higher levels of VEGF expression correlating with less severe gastric ulcers in murine models of stress-induced gastric ulceration (Malara et al. 2005). VEGF has also been shown to regulate normal vasculature in the small intestinal villi, with excessive inhibition resulting in regression of normal microvasculature, and reduced vascular density (Howdieshell et al. 2001; Saif et al. 2007; Pollom et al. 2015). Clinically, the inhibition of VEGF has been shown to delay healing of gastric erosions, reduce vascular density in the small

intestinal villi, and induce epithelial ulceration, supporting a role for VEGF in healing (Mangoni et al. 2012; Pollom et al. 2015). This regulatory role could be consistent with the timing of VEGF upregulation seen in the present study, occurring at the 15 week time-point in the colon only. At this time point, 9 weeks following the last radiation dose, we have clearly documented and reported on a transition from acute to chronic RIGT phenotype which is able to be seen histologically (Stansborough et al. 2017). This effect is likely due to alteration of the wound healing process, as well as vascular changes resulting in impaired delivery of healing agents to the damaged tissue (Mangoni et al. 2012). This study, in combination with findings of previous literature, suggests a role for VEGF in the progression to chronic RIGT, possibly playing a part in wound healing following acute RIGT. This potential role of VEGF in RIGT needs to be further explored, particularly with the increased study of anti-VEGF as adjuvant cancer therapies (Barney et al. 2013; Pollom et al. 2015). When VEGF inhibitors (VEGFIs) are combined with radiotherapy, gastrointestinal toxicity has been shown to significantly increase (Barney et al. 2013; Pollom et al. 2015). This potential for increased intestinal toxicity needs to be considered when determining the clinical potential of these agents.

Whilst TGFβ expression was significantly increased 3 weeks into the fractionated radiation schedule in our rat model of RIGT, consistent with previous literature, it was not significantly upregulated at 6 and 15 weeks, nor was it altered following irradiation of TAMECs (Richter et al. 1998; Wang et al. 2001). The lack of increase in TGFβ at the chronic RIGT time point was somewhat unexpected and in contrast with previous literature (Kleifeld et al. 2001). This may be explained by the different models used in the study by Wang and colleagues (2001, in which a scrotal hernia is surgically induced and the fractionated schedule uses higher doses over a shorter period of time (Wang et al. 2001). Our previous studies have shown fibrotic changes to the lamina propria and microvasculature at both the 6 and 15 week time points (Stansborough et al. 2017). With the complex interactions of TGFβ it is possible that the early upregulation of TGFβ, or alterations in the receptors of TGFβ are involved in these fibrotic changes. It is also unclear what the potential consequences of TGFβ involvement in RIGT may be, with

TGFβ being capable of opposing angiogenic effects, highly dependent on conditions and tightly regulated in the endothelium by the receptors ALK1 and ALK5 (Goumans et al. 2003). Whilst TGFβ binding with ALK5 has an anti-angiogenic effect, inhibiting endothelial cell migration and proliferation, ALK1 binding has the opposite effect (Goumans et al. 2003). Thus the ratio of ALK5 to ALK1 seems to be key to the functional role of TGFβ in pathological conditions. Further studies, both *in vitro* and *in vivo,* are needed to investigate the ALK5 to ALK1 ratio and determine where this balance lies in endothelial response to irradiation, and during RIGT, in order to determine the role of TGFβ in its pathobiology.

Angiostatin and endostatin immunostaining was shown to be significantly elevated in the lamina propria of both the jejunum and colon. This occurred only during the acute time points, with angiostatin increasing at 3 and 6 weeks, and endostatin at 3 weeks. The anti-angiogenic mediators angiostatin and endostatin, fragments of plasmin and type XVIII collagen, respectively, are responsible for inhibiting endothelial cell proliferation and migration, as well as inducing apoptosis in the case of endostatin (Ribatti 2009). Endostatin is also known to directly inhibit MMP-2 (Abdollahi et al. 2004). Although this current study is the first to assess angiostatin and endostatin in an animal model of RIGT, response of angiostatin to radiation has previously been assessed *in vitro* (14). Oh and colleagues (2014) finding angiostatin protein levels to be significantly increased in human breast tissue derived cancer endothelial cells following 4 Gy irradiation (Oh et al. 2014). Whilst endostatin has not yet been investigated in RIGT, Endostar, a recombinant human endostatin, has been shown to significantly decrease MMP-2, -9, -14, and VEGF immunostaining in nasopharyngeal carcinoma xenografts in mice following 6 Gy irradiation (Peng et al. 2012). Although the roles of angiostatin and endostatin likely differ in normal vs tumour tissue response to radiation, these studies, in combination with the present study, suggest a role of these anti-angiogenic mediators in radiation response, likely linked to the MMP pathway.

MMP-2 and -9 mRNA and protein expression was significantly increased following irradiation of TAMECs. The significant increase in MMP-2 expression is consistent with previous studies finding an increase in MMP-2 24 hours following 4 Gy irradiation of human normal endothelial cells and TAMECS (NECs and CECs, respectively) derived from breast tissue (Oh et al. 2014). However, the same study found MMP-9 not to be altered by irradiation of TAMECs in contrast to the findings of our current study (Oh et al. 2014). This is not entirely unexpected, due to species and tissue differences between the two studies, with MMP-9 regulation known to depend on cell type (Vincenti and Brinckerhoff 2007). Despite these conflicting results, the similarities between the studies highlight a role for MMP-2, in particular, in endothelial regulation following irradiation.

The increase in MMP-2 and -9 expression was also significantly attenuated, both at the mRNA and protein level, by the pre-treatment of TAMECs with SB-3CT. SB-3CT is the first mechanism-based MMP inhibitor, potently and selectively inhibiting MMP-2 and -9. The effect of SB-3CT is unique in its selectivity, as it forms a reactive species within the active sites of MMP-2 and -9 (Kruger et al. 2005) and was developed in response to a lack of efficacy of broad-spectrum, synthetic MMP inhibitors when used as anti-cancer therapies in clinical trials.

An unexpected finding in the present study was the significant decrease in mRNA expression of MMP-2 and MMP-9 following pre-treatment with SB-3CT in TAMECs following irradiation, as the mechanism of action of SB-3CT is in inhibiting activation. Additionally, SB-3CT significantly increased MMP-2 activity in TAMECs when combined with irradiation, having no effect on activity of MMP-9. The significant decrease in proMMP expression by SB-3CT is not unprecedented, with SB-3CT significantly decreasing proMMP-9 levels in a mouse model of transient focal cerebral ischemia (37). The study hypothesized this decrease was likely due to a positive feedback mechanism between MMP activity and gene transcription (Gu et al. 2005).

To our knowledge, this is the first study to assess mRNA expression of MMP-2 and -9 following SB-3CT administration. There are endogenous mediators that can be regulated by active MMP-2 and -9, such as TGFβ, and pharmacological reagents, such as doxycycline, that have been shown to regulate the mRNA expression of MMP-2 and -9 by altering mRNA stability (Yan and Boyd 2007). Whether SB-3CT is regulating transcription via a similar process, by affecting the promotor regions of these MMPs, or through disruption of a positive feedback mechanism, is unclear. The regulation of mRNA expression of both MMP-2 and MMP-9 by SB-3CT should be investigated further and current studies are underway in our laboratory.

The use of a single cell type in this study is a limitation as normal and tumour endothelial cells are phenotypically different and subsequently respond differently to irradiation; therefore it is likely that the response of normal gut endothelial to SB-3CT would differ to the findings of the present study (Oh et al. 2014). Thus, in order to further assess the possible effects of MMP inhibition in RIGT, future studies assessing SB-3CT in an *in vivo* model of RIGT are warranted. However, the findings of this study highlight a relationship between radiation, MMP expression and tumour endothelium. Due to the potential use of specific MMP inhibitors as cancer therapies, and with the involvement of both MMP's and the endothelium in the development and progression of RIGT, the findings of this study highlight the need for further research and consideration as to possible dual effects of MMP inhibitors in this setting (Strup-Perrot et al. 2006; Angenete et al. 2009; Stansborough et al. 2017).

Conclusion

The findings of this study support a role for the vascular mediators VEGF, TGFβ, angiostatin, and endostatin in RIGT following fractionated irradiation. Additionally, MMP-2 and -9 were shown to be involved in endothelial response to radiation *in vitro,* however inhibition by SB-3CT did not alter cell viability. The findings of the *in vitro* component of the present study elucidate the effects of SB-3CT on the endothelium, with unexpected findings of significantly inhibited proMMP expression, but not active

MMP expression, and confirm the role of MMP-2 in particular in the endothelial response to irradiation. These results highlight the need for further pre-clinical studies to assess the effect of MMP inhibitors and anti-angiogenic drugs in RIGT, particularly as these drugs are currently being investigated as adjuvant cancer therapies.

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Declarations of Interest

The authors report no declarations of interest.

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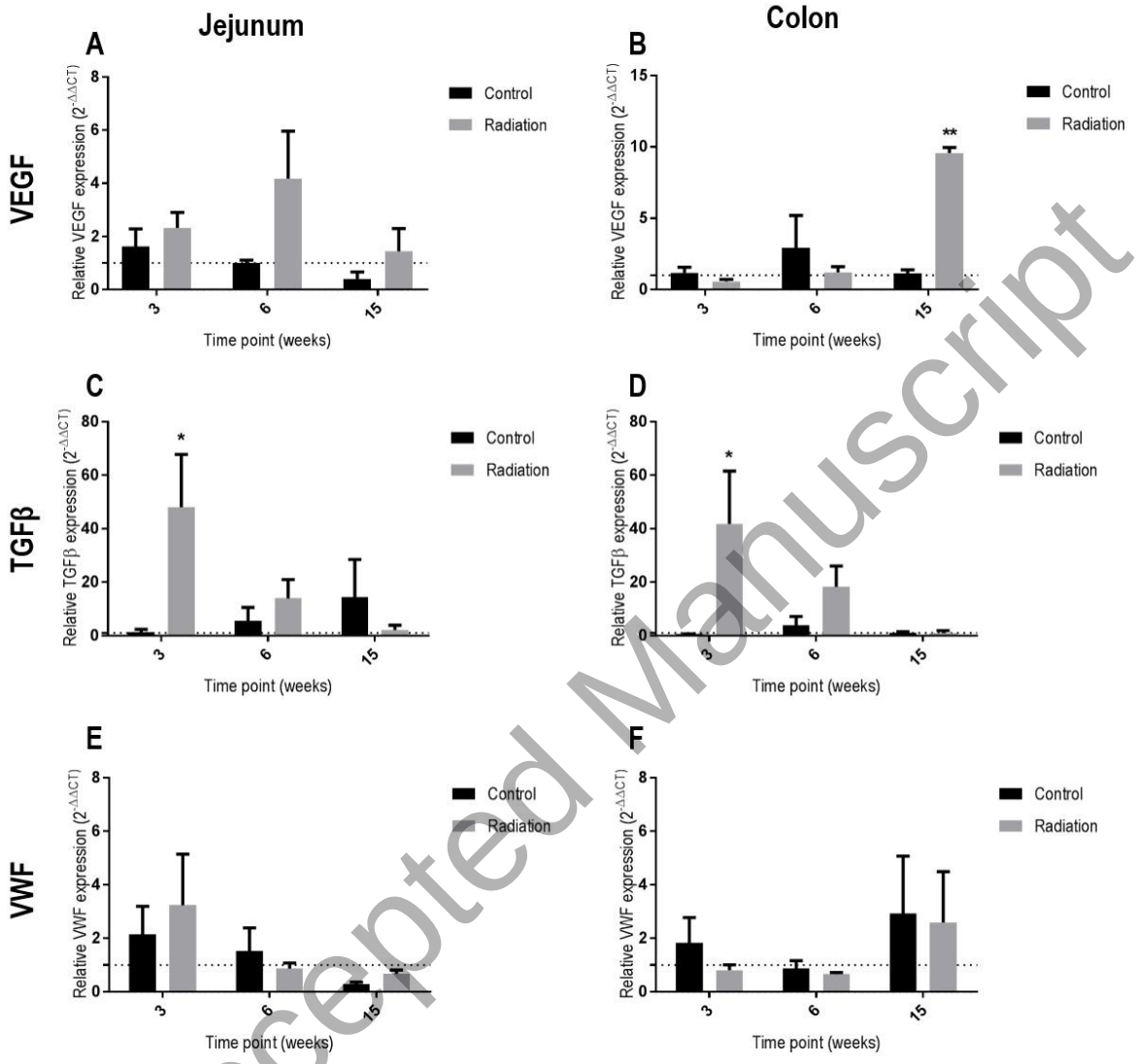
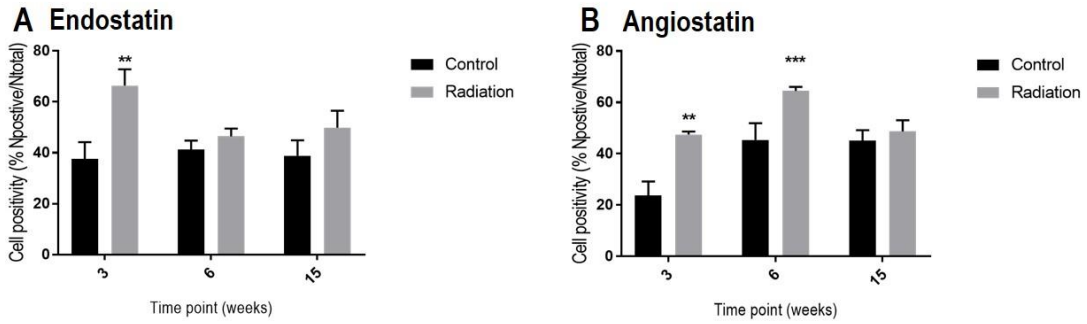
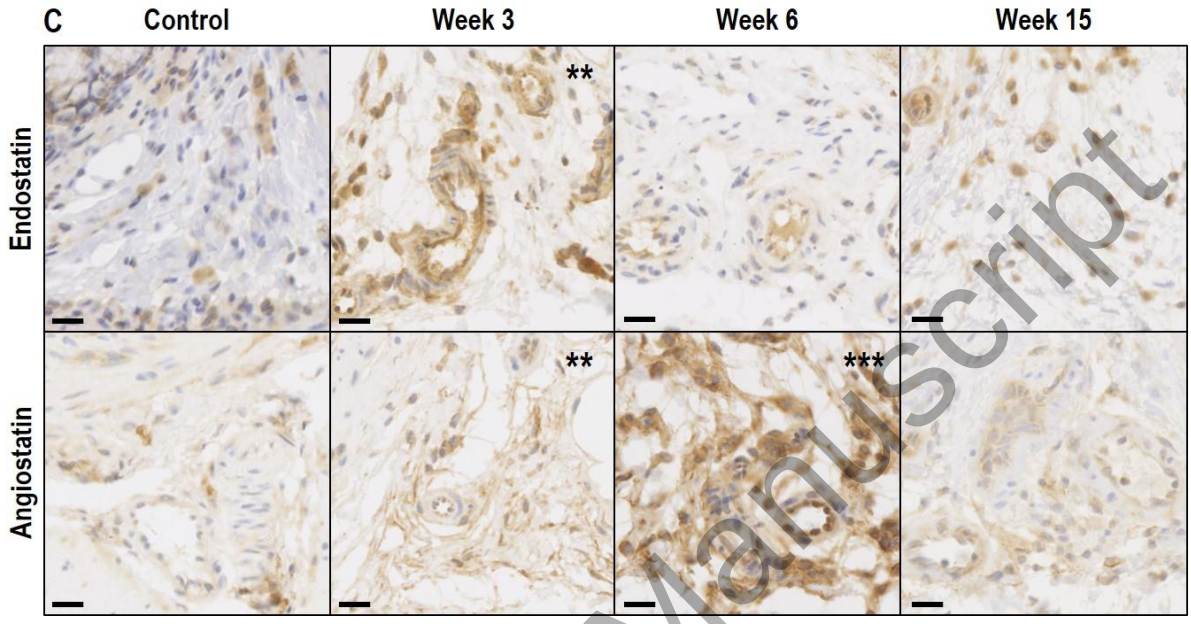
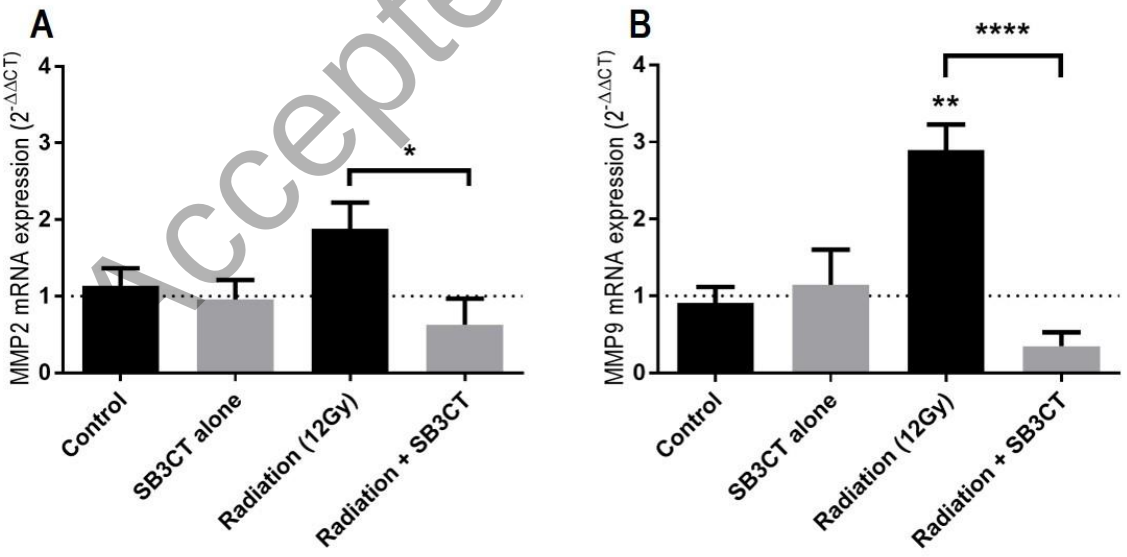
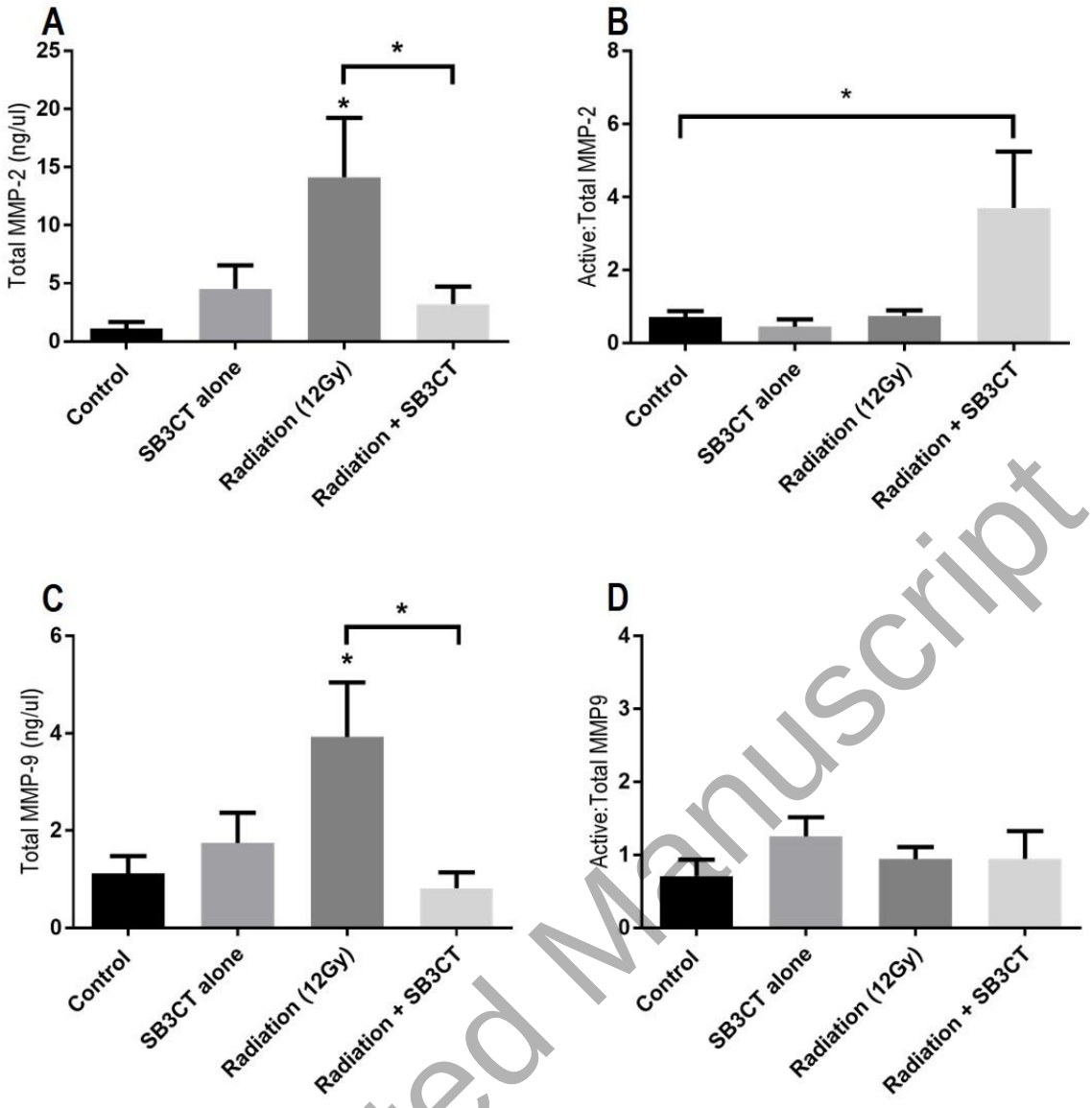
Supplementary Figure 1. Conformational staining of MMP-2, MMP-9, VWF, and VEGF in tumour-associated microvascular endothelial cells (TAMECs). 100x magnification

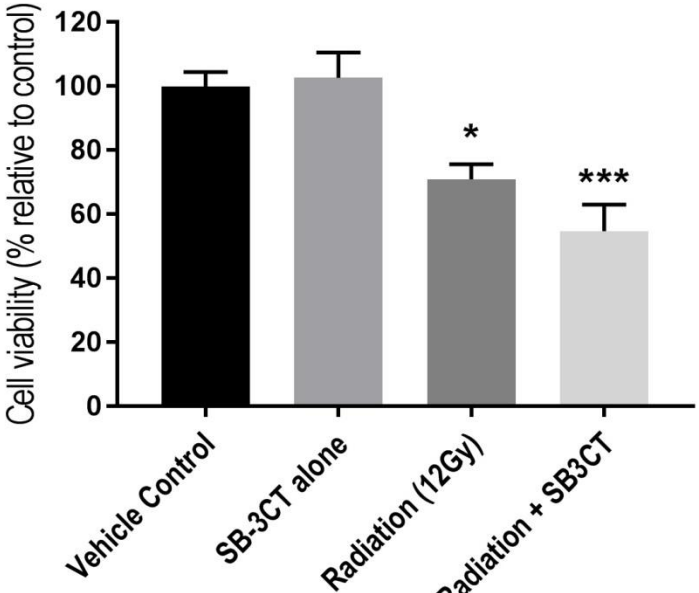












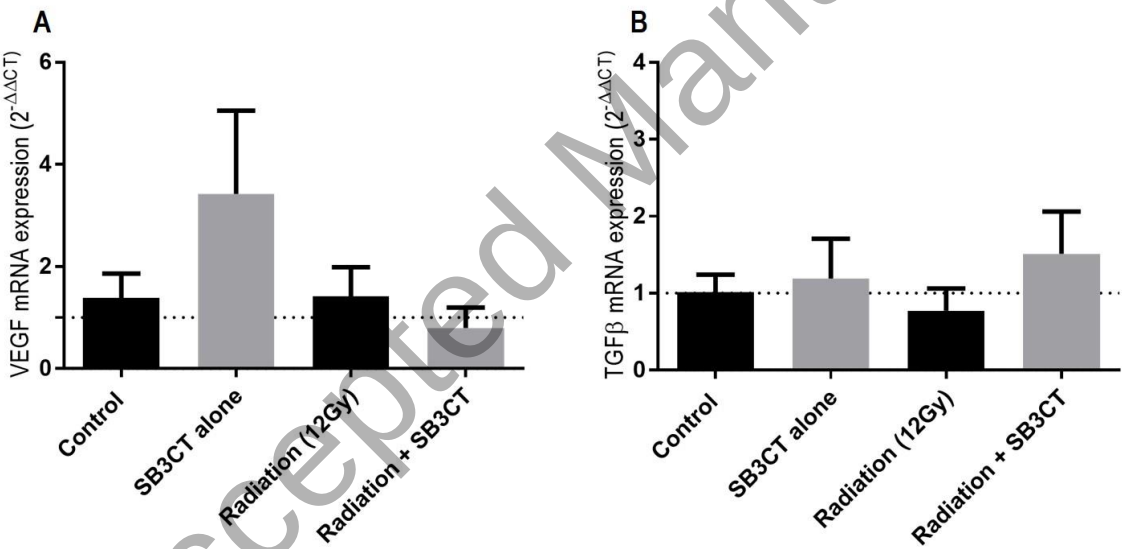


Table 1. 18S, VEGF, TGFβ, VWF, MMP-2, and MMP-9 primer sequences

Gene	Primer sequence (5'-3')	Nucleotide Position	Amplicon length (bp)	Tm (°C)	Ref
18S	**F** CATTCGAACGTCTGCCCTAT	344-452	109	60	(Uchida et al. 2010)
	R GTTTCTCAGGCTCCCTCTCC			60	
VEGF	**F** AGGCGAGGCAGCTTGAGTTA	1601-1766	166	62	(Zhang et al. 2014)
	R CTGTCGACGGTGACGATGGT			64	
TGFβ	**F** ATGACATGAACCGACCCTTC	897-1073	177	60	(Close et al. 2005)
	R ACTTCCAACCCAGGTCCTTC			60	
VWF	**F** GCCTCTACCAGTGAGGTTTTGAAG	4292-4587	296	63	(Boerma et al. 2004)
	R ATCTCATCTCTTCTCTGCTCCAGC			63	
MMP-2	**F** CTG ATA ACC TGG ATG CAG TGCT	2138-2272	135	55	(Al-Dasooqi et al. 2010)
	R CCA GCC AGT CCG ATT TGA			50	
MMP-9	**F** AAG CCT TGG TGT GGC ACG AC	760-876	117	56	(Vikman et al. 2007)
	R TGG AAA TAC GCA GGG TTT GC			52	