University of Alberta

Regulation of collagen type I production by ionizing radiation and transforming growth factor-β1 in primary human skin fibroblasts derived from early stage breast cancer patients in relation to acute radiation-induced toxicity

by

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Abstract

Regulation of collagen type I (CI) in fibroblasts is critical for the onset and development of skin toxicities induced by radiation therapy (RT). Transforming growth factor beta-1 (TGF-β1) and vascular endothelial growth factor (VEGF) promote CI synthesis, while its degradation is modulated by matrix metalloproteinase-1 (MMP-1), tissue inhibitor of MMP-1 (TIMP-1) and cathepsin K. We investigated the effects of ionizing radiation (IR) and/or TGF-β1 on CI levels and factors involved in CI regulation in primary skin fibroblasts derived from 40 early stage breast cancer patients. We determined statistical relationships between CI levels and acute skin toxicity events in the same patients after RT. We found that inhibition of collagenolytic activity by TIMP-1 affects CI levels in response to TGF-β1 and/or IR treatment of primary human fibroblasts, and increased patient-derived fibroblast CI levels in response to ex-vivo IR could be predictive of acute RT-induced toxicities in patients.

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List of Abbreviations

α-SMA	Alpha-smooth muscle actin
AJCC	American Joint Committee on Cancer
CTC	Common Toxicity Criteria
CTCAE	Common Terminology Criteria for Adverse Events
DMEM	Dulbecco's Modified Eagle's Medium
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
HRP	Horseradish peroxidase
IL-1	Interleukin-1
IL-6	Interleukin-6
IR	Ionizing radiation
LAP	Latency-associated peptide

LTGF-β1	Latent transforming growth factor beta-1
MAPK	Mitogen activated protein kinase
MMP-1	Matrix metalloproteinase-1
NIH	National Institutes of Health
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene fluoride
RT	Radiation therapy
RCT	Randomized controlled trials
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SNPs	Single-nucleotide polymorphisms
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TGF-β1	Transforming growth factor beta-1
TIMP-1	Tissue inhibitor of metalloproteinase-1
TMB	Trimethylbenzidine

TNF-α	Tumour necrosis factor-alpha
TNM	Tumour node metastasis
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A

Chapter 1. Introduction

Breast cancer is the most commonly diagnosed cancer among women. Globally, breast cancer accounts for 23% of all new cancer cases in women, and in 2008, breast cancer was the leading cause of cancer deaths among women at 14% [1]. In 2010, an estimated 27.6% of new cancer cases were breast cancer cases diagnosed in Canadian women, while 14.8% of all cancer-related deaths among women in Canada was due to breast cancer [2]. With the advent of regular and systematic screening, breast cancer can be detected at the early stage and with proper treatment may be potentially curable. Early breast cancer treatment modalities include breast conserving surgery, polychemotherapy, radiation therapy (RT), hormonal therapy and for a subset of patients, targeted therapy. The effectiveness of many of these treatments has been established through meta-analysis of randomized controlled trials (RCT) conducted by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) [3-6].

1.1 Radiation therapy (RT)

In early stage breast cancer patients, the ipsilateral breast recurrence after breast conserving surgery is significant with adjuvant RT. These recurrence events are due to the spread of residual microscopic disease unaffected by surgical intervention. RT, the standard of care after breast conserving surgery, is delivered with the purpose of eliminating microscopic disease and significantly reducing the risk of local recurrence. Meta-analysis of RCT comparing breast conserving surgery with and without RT showed a significant reduction of local recurrence in the adjuvant breast RT arm compared to surgery alone and the 5-year risk of local

recurrence was reduced by 19%. Breast cancer mortality was also shown to be affected as the 15-year risk of death from breast cancer was reduced by 5.4% [5]. The widely adopted standard RT regimen consists of a total dose of 50 Gy delivered in 25 daily fractions over 5 weeks followed by a boost of 5 fractions of 2 Gy delivered to the tumor site [7].

1.2 Acute radiation-induced toxicity and fibrosis

The mechanistic basis of RT was initially thought to rely on the cell killing effect of irradiation as characterized in the classic target-cell hypothesis [8]. In line with this theory, the appearance and severity of toxicity due to RT was presumed to be directly related to the radiosensitivity of a target cell type, whereby the loss of parenchymal cells and vascular endothelial cells was thought to result in the clinical manifestation of radiation injury [9]. It is now recognized that RT elicits a coordinated biological response during and beyond the early effects of direct cellular damage involving the release of cytokines and growth factors that initiate cytokine cascades leading to changes in local tissue function and structure [10]. These changes are often unintentional effects of therapy and could be dose-limiting factors during RT treatment. In early stage breast cancer treatment, they can be distinguished as acute and late adverse effects that manifest in the skin of treated patients. Within weeks of RT, early signs of epithelial damage appear as skin erythema, dry and moist desquamation, and hyper-pigmentation that are typically resolved over time. These side effects are categorized as acute toxicity by Common Terminology Criteria for Adverse Events (CTCAE) [11]. The majority of breast cancer patients treated with RT

experience a certain degree of acute radiation skin toxicity, which occurs within 90 days of the initiation of RT. Damage is incurred by radiation to dermal tissue causing pigmentation changes and erythema. The progression of RT increases radiation damage, depleting dermal stem cells resulting in moist and dry desquamation. Severity of acute skin reactions to irradiation varies depending on treatment-related factors, such as dose size and total dose delivered, and also patient-dependent factors such as breast size and age of the patient [12].

At the molecular level, radiation-induced toxicities can be paralleled to an excessive wound healing response to radiation injury. Damage to epithelial cells of the skin by both direct radiation and the resulting increase in reactive oxygen species (ROS) induce a nearly immediate release of inflammatory signals, triggering a cascade of events involving platelet activation, increased blood vessel permeability, and recruitment of macrophages and other cells involved in the wound healing response. In a healthy wound response, these cells in turn produce signaling molecules that modulate the synthesis of a scaffold of provisional matrix proteins that eventually facilitate the resolution of the wound by the expansion of new epithelial cells creating healthy epithelium over the area of radiation-induced damage [9, 13].

After months and possibly years of an apparent though misleading latent period, a time during which the aforementioned cytokine cascades persist undetected, late effects of RT are eventually expressed. The chronic and potentially debilitating manifestation of fibrosis of the skin is such a late effect.

Fibrosis is a long-term dynamic process characterized by prolonged

fibroblast activation and tissue remodelling, affecting up to 40% of women with early stage invasive breast cancer who received RT [14]. The latter phase of radiation-induced fibrosis deviates from the healthy wound healing paradigm by a prolonged overabundance of inflammatory cytokines causing a sustained inflammatory response accompanied by continuous tissue remodelling. The ECM homeostasis is thus propelled towards matrix protein synthesis resulting in matrix accumulation and eventual fibrotic scar formation [15]. Early studies involving pig skin models found that irradiation drastically increased the expression of key pro-fibrotic cytokines such as transforming growth factor (TGF)-β1 [16] while reducing ECM degradation by greatly decreasing proteases such as metalloproteinase-1 (MMP-1) and increasing synthesis of tissue inhibitor of MMP-1 [17]. A previous study using pig skin model reported a 10-fold increase in collagen accumulation, a major component of the ECM, in irradiated tissue compared with healthy tissue [18]. The main cellular component responsible for the synthesis of the majority of ECM proteins in tissue remodelling, the fibroblast, has become a major focus in the search for potential predictive markers of radiation-induced toxicity.

1.3 Predictive markers for radiation-induced injuries

1.3.1 The fibroblast

Fibroblasts are widely distributed mesenchymal cells that comprise the main cellular component of the connective tissue [19]. This relative abundance of fibroblasts is evidence of one of its major functions: the synthesis of ECM proteins. The ECM is a conglomerate of an elastic network of collagens (mostly

collagens type I and III), proteoglycans, and glycoproteins, forming the structural components of connective tissues [20]. Besides its obvious structural function, the ECM also acts as a communication matrix for the cells of the connective tissue, forming localized signaling environments while allowing diffusion of molecular cues. A major role of the ECM is to bind cytokines, creating a concentrated reservoir of signaling molecules around receptive cells to enable immediate molecular responses following certain stimuli [21, 22]. Other key functions involving fibroblast mediation include epithelial differentiation, regulation of inflammation, and wound healing [23]. Defining fibroblasts in terms of molecular markers is difficult, as the molecular definition of fibroblasts is tissue dependent. In the skin, fibroblasts are commonly identified by the surface marker desmin, while in cardiac tissue fibroblasts express the collagen receptor discoidin-domain receptor 2; these molecules are not specific to fibroblasts. Thus, there is a notable lack of a single dependable molecular marker that distinguishes fibroblasts from other cell types [24].

Fibroblasts contribute to many aspects of the normal wound healing process. At the onset of injury, physical stress to the wounded tissue is a strong stimulus for fibroblast activation [25], making fibroblasts sensitive injury detectors. During radiation-induced injury, fibroblasts can also take on the role of injury detection indirectly, by responding to cytokine stimuli released from reservoirs within the ECM caused by radiation-induced ROS generation. The process of early recruitment of monocytes and macrophages involves local fibroblasts within the wound area in the role of secretion of cytokines and

chemokines. This role continues during the subsequent inflammatory response. These signaling molecules induce synthesis of additional cytokine signals from surrounding cells in paracrine fashion while also acting as autocrine signals on fibroblasts themselves. This results in additional cytokine production, activation of fibroblasts, and ultimately, and critically during the healthy wound repair process, the terminal differentiation and apoptosis of fibroblasts [26]. The transient activity of fibroblasts is responsible for the deposition of provisional ECM, facilitating healthy wound resolution [27].

In chronic wounds such as radiation-induced fibrosis, fibroblasts are similarly involved in wound healing initiation, as in normal wound healing. However, as the process continues with prolonged inflammation, fibroblast cytokine production contributes to the perpetual cytokine cascades that plague chronic wounds, feeding into prolonged and sustained inflammatory response through constant autocrine activation and paracrine promotion of activity from other inflammatory cell types. Notably, fibroblasts are activated but are not ultimately driven to apoptosis by cytokine signals. Instead, fibroblasts are maintained in their activated state, thus sustaining activity. The most integral effect of fibroblasts in the development of chronic wounds is the continuous deposition and accumulation of ECM proteins, eventually leading to permanent scar formation [13].

Due to the central importance of the ECM synthesis by fibroblasts in radiation injury, direct links between radiation exposure and fibroblast response have been proposed. In the late 1980s and early 1990s, numerous investigations

were carried out searching for a direct correlation between *in vitro* radiosensitivity of human skin fibroblasts as detected by clonogenic survival assays and the late radiation-induced effect of fibrosis [9]. What followed was a series of conflicting results generated over a number of years, beginning with earlier studies reporting positive correlations linking normal human skin fibroblast radiosensitivity with the occurrence of radiation-induced fibrosis. These studies, while showing significant correlations with the late effect of RT such as fibrosis, did not identify a significant correlation between normal fibroblast radiosensitivity and high grade acute radiation effects such as erythema and moist desquamation. They also suggested that validation within larger groups of patients was warranted [28, 29]. As investigations progressed with larger groups of patients, conflicting results arose regarding the existence of correlation between normal skin fibroblast radiosensitivity and occurrence of late radiation-induced fibrosis. Some of these conflicting data were generated by studies involving breast cancer patients and showed that while differences in intrinsic radiosensitivity existed amongst primary human fibroblasts, this did not directly account for different patient outcomes in radiation-induced toxicities. Thus, radiosensitivity may be inferior to other biological factors as a predictive marker of radiation-induced acute or late injuries [30, 31].

1.3.2 Cytokines as markers of radiation-induced injury

Cytokines are intracellular signaling proteins that mediate cellular function and include growth factors, adhesion molecules, and chemokines. These signaling molecules are integral to cellular and tissue response to harmful stimuli

such as radiation, modulating inflammatory responses and the wound healing process. Due to the ability of cytokines to alter cellular response and initiate changes in tissue structure and function, it has been proposed that cytokines may possibly serve as valuable predictive markers for radiation-induced injuries [32]. The cytokines that have attracted significant interest as predictive markers include interleukins -1 (IL-1) and -6 (IL-6), fibroblast growth factor-2 (FGF-2), and TGF-β1. The growth factor that has been the focus of the bulk of investigations into predictive cytokine markers of radiation-induced toxicities is TGF-β1. Extensive work has been conducted in pulmonary models of radiation-induced toxicity attempting to find correlative evidence linking circulating plasma TGF-β1 levels in lung cancer patients before, during, and after RT to the occurrence of radiation injury. The results of these correlative studies have been conflicting. Studies have reported elevated plasma TGF-β1 levels at the end of radiotherapy to be useful predictive markers for radiation-induced pulmonary inflammation in non-small cell lung cancer patients [33]. Later work by a number of research groups reported that this predictive value did not exist. There was no significant trend linking plasma TGF-β1 concentrations at any point during radiation treatment to development of radiation-induced pulmonary injuries [34]. Studies involving plasma TGF-β1 levels in breast cancer patients have also been conducted, showing some evidence of predictive value [35], but these studies remain inconclusive. More recent work has investigated the *TGF-β1* gene as a predictive marker by conducting correlative investigations between late radiation-induced fibrosis and single-nucleotide polymorphisms (SNPs) in the *TGF-β1* gene.

Though earlier reports have suggested links between certain SNPs within the *TGF-β1* gene and enhanced risk of fibrosis, contradictory evidence has surfaced refuting previous results [36, 37]. In light of the contradictory evidence of the predictive value of differences in plasma levels and genetic differences in *TGF-β1* among patients, it is reasonable to assume that TGF-β1 alone may not be the most valuable predictor of radiation-induced toxicities.

1.4 Collagen type I

Since fibroblasts produce ECM proteins, factors involved in this process may hold a predictive value for the development of acute and/or late radiation toxicities. The human genome includes 42 genes that encode unique collagen subunits called α-chain molecules. These α-chains combine into higher order triple helixes to form homo- or heterotrimeric molecules of collagen. So far, approximately 40 different combinations of α-chains have been identified as different collagen types creating a diverse collagen protein family [38]. Of the various members of the collagen family, collagen type I is the most abundant in skin, comprising 80-85% of all collagen types in the dermal matrix [39]. Collagen type I is classified as a fibrillar collagen due to its innate tendency to form highly organized higher order fibre structures [20]. These tightly ordered collagen fibres are the source of the elasticity and tensile strength that characterize the skin.

Collagen type I is composed of two proα1(I) α-chains and one proα2(I) α-chain [40]. Following translation of the α-chain subunits, the separate α-chain units combine into a heterotrimeric pre-procollagen molecule, which then

undergoes a series of post-translational modifications as it is transported from the cell endoplasmic reticulum, through the Golgi apparatus, and into secretory vesicles. The molecule secreted from the fibroblast is a procollagen type I molecule. In order to produce mature collagen type I, proteases within the extracellular environment must cleave off the carboxy- and amino-terminal propeptides of collagen type I [41]. The mature collagen type I molecule is then associated with other units of collagen type I and other fibrillar collagens to form heterogeneous collagen fibrils, which are deposited and incorporated into the ECM [20].

During normal wound healing, synthesis and deposition of collagen type I is crucial for the formation of a temporary scaffold to allow for wound closure, epithelial cell expansion, and wound resolution. In parallel, the importance of fibroblast deposition of collagen type I in the adverse process of radiation-induced fibrosis cannot be understated. Increased collagen type I synthesis has been documented in studies involving breast cancer patients undergoing RT. Previous studies showed increased collagen type I synthesis in irradiated skin compared to contralateral healthy skin using mRNA and interstitial fluid concentrations of propeptides of collagen type I [42-44]. In an early porcine model, it was noted that 6-15 months following irradiation large areas of fibrotic scar tissue formed in which protein expression and mRNA expression of collagen type I was increased compared to healthy tissue [18]. In breast cancer patients, increased amounts of collagen type I has been found in irradiated tissue compared to non-irradiated contra-lateral tissue years after RT treatment [45]. Fibroblasts from irradiated skin

biopsies of 5 patients undergoing RT produced higher levels of procollagen type I, compared to fibroblasts isolated from non-irradiated tissue [46]. In light of these and other findings, fibroblasts have a central role in collagen type I production and the initiation, progression, and persistence of acute radiation-induced toxicities and fibrosis.

1.5 Fibroblast activation/the myofibroblast

At the onset of injury, including radiation-induced injury, local fibroblasts within the wound site receive a plethora of stimuli that may include mechanical stress, oxidative stress, and exposure to the release of reservoirs of signaling molecules from the surrounding ECM [47]. In response to these stimuli, fibroblasts will undergo a transition to an activated state, characterized most commonly by the expression of α-smooth muscle actin (α-SMA), a molecular feature common to myocytes. These activated smooth-muscle-like fibroblasts are termed 'myofibroblasts' [48]. First observed in granulation tissue of healing wounds [48], myofibroblasts further expand the role of fibroblasts in wound repair. The expression of α-SMA in cytoskeletal fibrils [26] provides the contractile force required in wound healing for wound closure [23]. Myofibroblasts also generate cytokines and growth factors during the inflammatory response, and most importantly, activation of fibroblasts to the myofibroblast phenotype greatly increases synthesis and secretion of collagen type I and other ECM proteins [26].

1.6 Regulation of collagen type I synthesis

1.6.1 TGF-β1

TGF-β1 is a member of a superfamily of proteins including activins, inhibins, bone morphogenetic proteins, and growth and differentiation factors [49]. Three highly homologous isoforms of TGF-β (1 through 3) exist in mammals. These molecules regulate a number of biological processes including cell growth, differentiation, apoptosis, cell migration, immune cell function, and ECM production [50]. TGF-β1 is a key regulator of wound healing and the wound response. An early event at a site of injury is the degranulation of TGF-β1-rich platelets, which releases a significant influx of TGF-β1 inundating the wound site stimulating inflammatory cell recruitment, fibroblast recruitment, and subsequent TGF-β1 synthesis by these cell types. The involvement of TGF-β1 has been demonstrated in impaired wound healing models where topical application of TGF-β1 improved healing [51]. TGF-β1 promotes ECM deposition by enhancing ECM synthesis through induction of several collagen α-chain gene promoters including COL1A1, COL1A2, COL3A1, COL5A2, COL6A1 and COL6A3 as well as inhibiting ECM degradation through down-regulation of ECM-degrading proteases [52, 53]. The contractile activity of myofibroblasts in wound healing is also enhanced by TGF-β1 [54], further emphasizing the critical role of fibroblasts in wound healing.

TGF-β1 is secreted as a pro-protein homodimer complex non-covalently associated with a dimer of peptide segments called the latency-associated peptide (LAP) [55]. The interaction between LAP and TGF-β1 (latent TGF-β1, LTGF-β1)

sequesters TGF-β1 in an inactive state and must be disrupted to release the active TGF-β1 molecule. Usually, LTGF-β1 is secreted as part of a larger complex that includes another protein called the LTGF-β-binding protein (LTBP) that serves to bind TGF-β1 to the ECM, forming a store of potentially active TGF-β1 [56]. In this way, TGF-β1 latency acts as a layer of regulation that dictates TGF-β1 activity as well as localization of that activity, modulating autocrine and paracrine signaling. TGF-β1 activation can be achieved by a variety of stimuli. Heat and changes in pH can alter the conformation of the LTGF-β1 complex thus releasing active TGF-β1, though this form of activation may not be relevant in most physiological situations. Proteolytic activity by proteases such as plasmin and matrix metalloproteinases (MMPs) can cleave TGF-β1 from the latency complex. Interactions with proteins that alter LTGF-β1 conformation, such as thrombospondin, can also activate TGF-β1 [56, 57]. In terms of radiation, TGF-β1 activation can be achieved by interaction with ROS. It has been previously shown that active TGF-β1 levels rapidly increased after irradiation in a murine mammary model [58]. The same research group also proposed that the latent complex of TGF-β1 is activated through conformational changes induced by oxidation by ROS [59]. The chronic condition of the radiation-induced fibrotic process may be enhanced by the activity of ROS due to the induction of ROS production by TGF-β1, forming a perpetuating positive feedback loop [22].

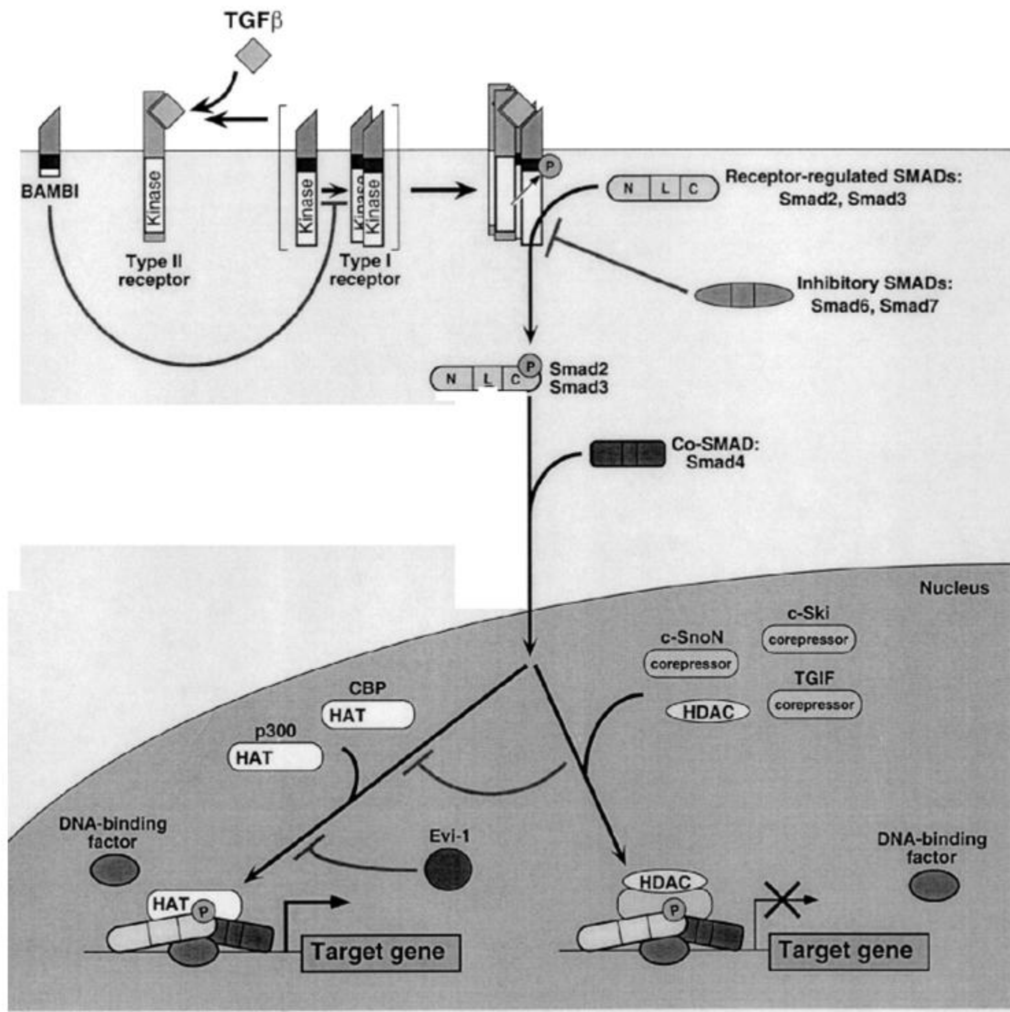


Figure 1. Transforming growth factor-beta (TGF-β) induced Smad3 signaling. TGF-β binding to the type II receptor initiates formation of a type I/II receptor complex that transmits an intracellular signal through interactions with Smads including Smad3. The resulting Smad complex migrates to the nucleus to act as transcriptional mediators, promoting synthesis of gene products such as collagen type I. Adapted from Kretzschmar, M., *Transforming growth factor-beta and breast cancer: Transforming growth factor-beta/SMAD signaling defects and cancer.* Breast Cancer Res, 2000. 2(2): p. 107-15.

Once active, TGF-β1 interacts with target cells through cell surface serine/threonine kinase receptors. The TGF-β1 ligand binds to a constitutively active type II serine/threonine kinase receptor, which recruits a type I receptor to the complex to be phosphorylated and activated by the type II receptor. The intracellular signaling molecule Smad3 is recruited to the activated type I receptor and phosphorylated. Smad3 is then released to form a complex with other Smad proteins that eventually translocate to the nucleus and activate transcription of several genes [50, 56] (Fig. 1). The involvement of intracellular signaling through Smad3 is responsible for many of the activities of TGF-β1 in fibroblasts, including induction of ECM synthesis, fibroblast activation, and autocrine TGF-β1 synthesis. In human skin fibroblasts, Smad3 signaling has been implicated in the induction of *collagen type I* gene transcription by TGF-β1 [60]**.** Synthesis of TGF-β1 through Smad3 intracellular signaling may be a major factor in the process of radiation-induced fibrosis. Smad3 involvement in TGF-β1 signaling in a murine skin fibrosis model has been reported, as Smad3 knockout models exhibited reduced fibrotic effects after bleomycin treatment [61].

TGF-β1 affects many aspects of the process of acute radiation-induced toxicities and fibrosis. As noted above, TGF-β1 is activated by RT through increased ROS following radiation. In fibroblasts, TGF-β1 induces ECM and collagen type I synthesis [60, 62], inhibits ECM-degrading proteases [63], and promotes fibroblast activation into myofibroblasts [34, 64]. Due to its pro-fibrotic effects, TGF-β1 may have a key role in the regulation of collagen type I

and the initiation and promotion of acute radiation skin toxicity and/or radiation-induced fibrosis.

1.6.2 VEGF

Vascular endothelial growth factor (VEGF-A) is a homodimeric glycoprotein first described as a diffusible endothelial cell-specific mitogen unique in its ability to induce vascular permeability [65, 66] and is now known to have roles in mediating cell proliferation, survival, and migration (Fig. 2). This observation led to the identification of the most important role of VEGF: the regulation of angiogenesis. VEGF-A is a member of a family of proteins including VEGF-B, VEGF-C, VEGF-D, and placental growth factor known to regulate growth of vascular endothelial cells, promote vascular permeability and extravasation of blood-borne components [67]. VEGF is expressed in a variety of cell types, including fibroblasts. Importantly, VEGF is a key factor in the initiation and progression of a healthy or fibrotic wound healing response. The process of inflammation that accompanies most fibrotic wound healing responses requires VEGF activity to induce vascular permeability, which allows access of the blood-borne inflammatory cells to the wound tissue. VEGF further contributes to the fibrotic response by promoting the mobilization of hematopoietic and endothelial progenitor cells [68], and providing a chemotactic signal for monocytes [69]. Mobilization of progenitor cells by VEGF has been shown in a murine model in which low dose radiation induced VEGF in conjunction with MMPs and promoted migration of progenitor cells [70]. These activities of VEGF may support the fibroblast and myofibroblast population at the wound site

by promoting the migration and incorporation of blood-borne bone marrow-derived fibroblast progenitors, thus increasing the population of collagen type I-producing cell types and promoting formation of fibrotic tissue.

Induction of VEGF expression has been linked to irradiation as mentioned above in murine models. *In vitro* studies have suggested that radiation induce VEGF through a mitogen activated protein kinase (MAPK) signaling pathway [71]. VEGF expression is induced by TGF-β1 in both healthy fibroblasts and fibroblasts isolated from fibrotic tissue [72]. An intracellular Smad 3 signaling pathway for this induction has been shown in a human pulmonary model, identifying TGF-β1 activating the pathway and inducing VEGF production [73]. The effects of irradiation and TGF-β1 on VEGF expression modulate regulation of collagen type I synthesis within a radiation wound site, contributing to the progression of the radiation-induced toxicity process.

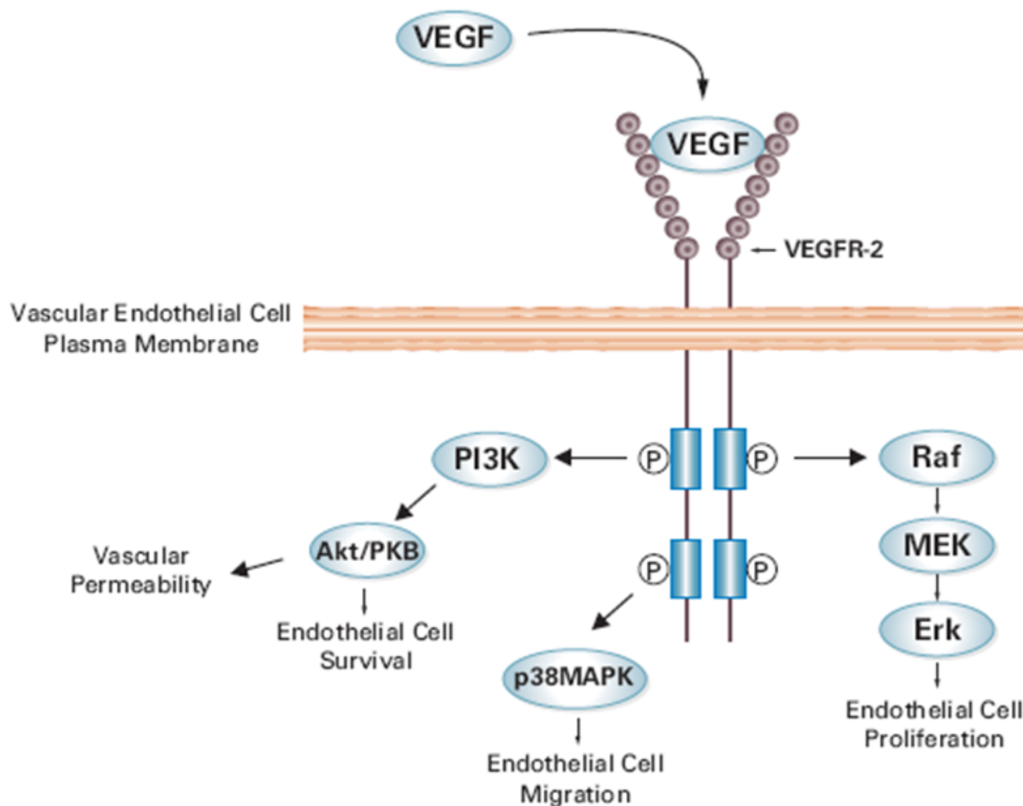


Figure 2. Signaling pathways activated by vascular endothelial growth factor (VEGF). VEGF binding to the VEGF receptor initiates intracellular signaling pathways leading to induction of responses including vascular permeability, cell migration, and cell survival. Rini, B.I. and E.J. Small, *Biology and clinical development of vascular endothelial growth factor-targeted therapy in renal cell carcinoma.* J Clin Oncol, 2005. **23**(5): p. 1028-43.

1.7 Regulation of collagen type I degradation

1.7.1 MMP-1 and TIMP-1

In normal wound healing and tissue remodelling, the deposition of collagen type I and ECM is balanced by their degradation by proteases, such as MMP-1. MMPs are a family of 25 endopeptidases that incorporate a Zn^{2+} or Ca^{2+} ion into their active sites [74, 75]. MMP-1 is produced by a variety of cell types including fibroblasts, and is secreted as a zymogen that must be activated by proteolytic cleavage involving other MMPs. Based on their primary substrate, MMPs are divided into a number of classes including collagenases, gelatinases, stromelysins, and others [63]. MMP-1, or interstitial collagenase-1, is among a few MMPs capable of degrading fibril-forming collagens, including collagen type I. The triple helix of collagen type I is cleaved by MMP-1 resulting in unwinding of the α-chains, making collagen type I more susceptible to further degradation [76]. The decreased ability of MMP-1 to degrade fibrillar collagen type I has been reported in fibrosis. As shown in a murine hepatic model, fibrosis was not resolved in mice expressing a mutant cleavage-resistant collagen type I [77]. In contrast, fibrolysis was achieved by over-expression of MMP-1 in another murine hepatic model [78]. Degradation of collagen type I and other collagens and ECM components by MMP-1 and other MMPs and their ability to process signaling molecules is important throughout the wound healing process. MMPs can be involved in the initiation of wound healing by releasing cytokines bound to the ECM, resulting in cytokine cascades. Chemokine activity can be greatly modulated by MMP-1 activity through direct proteolysis or creation of chemokine

gradients, dictating the influx of inflammatory and repair cells into the wound site [74]. Additionally, MMPs play a role in the process of epithelial to mesenchymal transition (EMT), which is a source of fibroblasts and myofibroblasts in wound healing. MMPs cleave epithelial cell interactions thereby allowing mobilization of epithelial cells in the process of EMT [79]. In relation to MMP-1 in the environment of a radiation wound, radiation has been observed to repress MMP-1 expression [17]. TGF-β1 also represses MMP-1, and as reported in a dermal fibroblast model, this repression once again involves TGF-β1 activated Smad3 signaling [80].

After extracellular activation of MMPs, their activity is regulated by the binding of tissue inhibitors of metalloproteinases (TIMPs). To date four TIMPs have been identified (TIMP-1, TIMP-2, TIMP-3, TIMP-4), with TIMP-1 serving as the inhibitor of MMP-1 among other MMPs. TIMPs inhibit MMPs by directly binding, similar to substrate binding, to the Zn^{2+} or Ca^{2+} active sites in a 1:1 inhibitor to enzyme ratio [81]. Regulation of TIMP-1 by both irradiation and TGF-β1 seems to directly oppose MMP-1 activity in the environment of a radiation injury. Irradiation has been shown to up-regulate TIMP-1 [17], while TGF-β1 is also known to drastically up-regulate TIMP-1 in human fibroblasts [82]. The regulation of MMP-1 and TIMP-1 upon radiation and TGF-β1 exposure suggests a significant shift in the balance of collagen type I synthesis and degradation.

1.7.2 Cathepsin K

Degradation of collagen type I in the regulation of ECM homeostasis is not limited to the extracellular environment. Intracellular collagen type I degradation is mediated by cathepsin K, previously described as the most potent collagen-degrading protease [83]. Cathepsin K is a cysteine endopeptidase, a member of a family of more than 20 cysteine, serine, and aspartyl proteases [84]. First described in osteoclast mediation of bone resorption, cathepsin K is unique among collagen-degrading proteases due to its ability to cleave collagen type I at multiple sites of the collagen triple helix [83]. Cathepsin K is mostly restricted to lysosomes where the acidic environment is optimal for its activity. This compartmentalization of cathepsin K has been reported in human fibroblasts [85]. Cathepsin K is synthesized as a zymogen that requires enzymatic cleavage, often by another mature cathepsin molecule, to gain activity. Along with the requirement for a low pH environment, the zymogen stage regulates cathepsin K activity. Another facet of cathepsin K regulation relates to the radiation injury event; ROS inhibits cathepsin K activity, which indicates another pro-fibrotic aspect of radiation [86]. In human dermal fibroblasts, cathepsin K expression as detected by staining with a polyclonal antibody has been observed in fibroblasts isolated from fibrotic tissue. In contrast, healthy fibroblasts did not express or expressed very low levels of inactive cathepsin K [87]. Collagenolytic activity of cathepsin K in fibroblasts is proposed to involve collagen type I internalization through endocytosis. During this process extracellular collagen type I fibrils deposited in the ECM are first fragmented by MMP-1 before being engulfed by

fibroblasts and translocated to lysosomes for cathepsin K-mediated degradation [88]. It has been found that TGF-β1 down-regulates murine cathepsin K in pulmonary and human dermal fibroblast models [85, 89], which suggests that TGF-β1 activation and subsequent induction after radiation injury may further unbalance the collagen type I deposition/degradation homeostasis.

1.8 Model of collagen type I regulation

The balance between collagen type I synthesis and its degradation in fibroblasts is a key element during the detrimental process of radiation-induced acute skin toxicity and fibrosis. Regulation of collagen type I expression in human skin fibroblasts is mediated by a number of cytokines and growth factors, the most potent of which is TGF-β1, referred to as the "master switch" in the process of fibrosis partly due to its capacity to activate and simulate fibroblast collagen type I synthesis [15]. Beyond this direct effect on collagen type I synthesis, the pervasive effects of TGF-β1 include interactions with a range of other regulators of collagen type I homeostasis in human skin fibroblasts. Following radiation, the promotion of vascular permeability by VEGF, which is induced by TGF-β1 [73], facilitates the migration of cell types involved in wound healing in response to chemotactic signals. Collagen-producing cells and fibroblast precursors are among the cell types that migrate to the wound site, contributing thereby to collagen type I synthesis as wound healing progresses. MMP-1 and its inhibitor TIMP-1 are also directly regulated by TGF-β1 [80]. Expression of cathepsin K, the intracellular collagen type I degradation mediator, is similarly influenced by TGF-β1 [89] (Fig. 3). Hence, the effect of IR and/or TGF-β1 on different factors

involved in collagen type I homeostasis in primary human skin fibroblasts may reflect how patients will respond to RT.

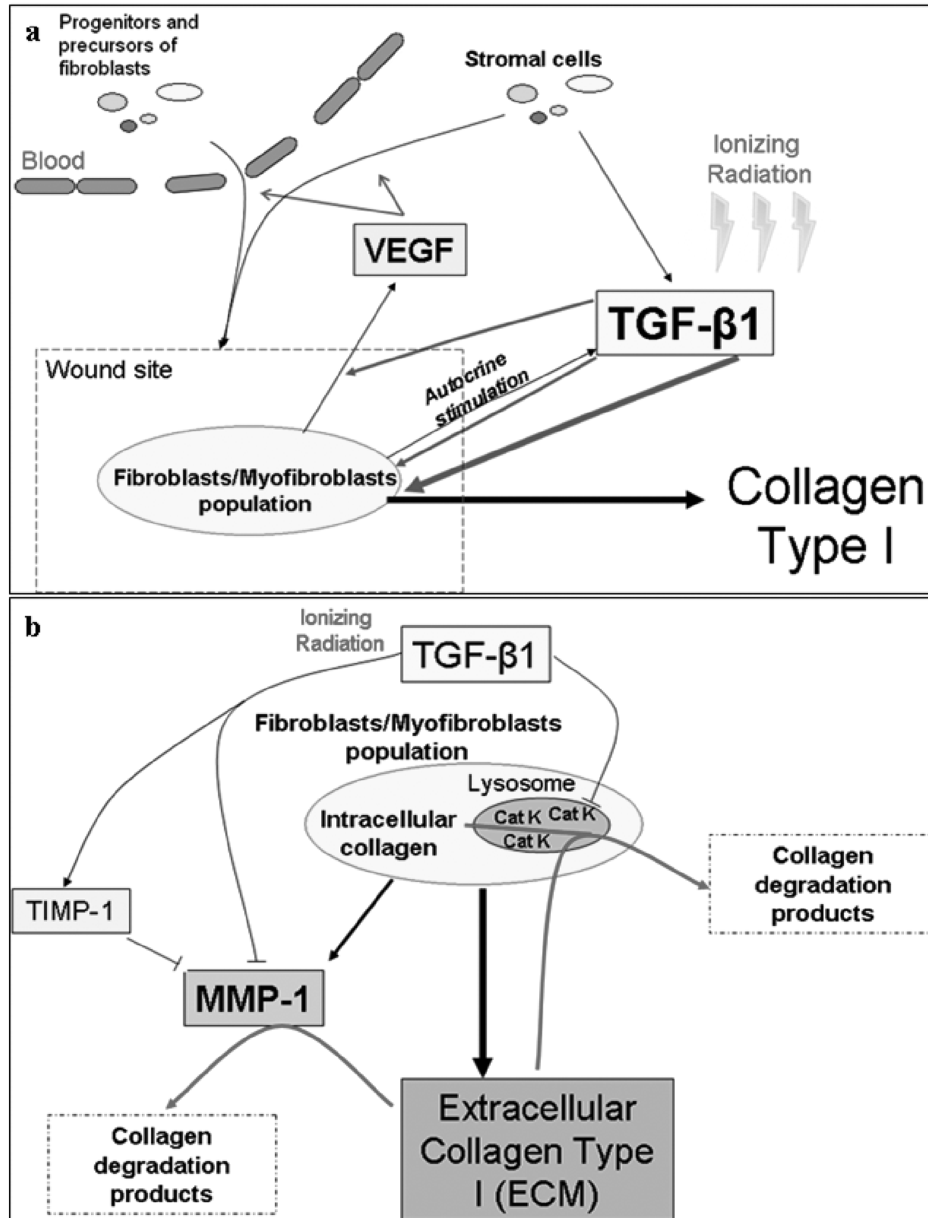


Figure 3. Model of collagen type I regulation in skin fibroblasts. (a) TGF-β1 activated by IR stimulates additional fibroblast TGF-β1 secretion as well as VEGF secretion. VEGF promotes migration and integration of stromal cells ie. fibroblasts and fibroblast progenitors from circulation into the wound site. This fibroblast/myofibroblast population produces collagen type I in response to TGF-β1 stimulation. (b) MMP-1 mediates extracellular collagen type I degradation and is inhibited by TIMP-1. TGF-β1 and IR inhibit fibroblast MMP-1 secretion and TIMP-1 secretion. Cathepsin K mediates intracellular collagen type I degradation. TGF-β1 inhibits cathepsin K expression. TGF-β1=transforming growth factor-β1, IR=ionizing radiation, VEGF=vascular endothelial growth factor, MMP-1=matrix metalloproteinase-1, TIMP-1=tissue inhibitor of metalloproteinase-1, Cat K=cathepsin K.

1.9 Statement of Hypothesis and Aims

Collagen type I and its regulation in human skin fibroblasts is central to the process of wound healing and the pathological processes leading to acute skin toxicities and later fibrosis in early stage breast cancer patients treated with RT. TGF-β1 is a potent inducer of collagen type I synthesis, and VEGF will further enable migration of potential collagen-producing cells into the wound site. Degradation of collagen type I is regulated by the extracellular collagenolytic activity of MMP-1 and its inhibitor TIMP-1, as well as intracellularly active cathepsin K, a potent collagen type I degrading protease. We hypothesize that the onset of radiation-induced toxicities in early stage breast cancer patients could be related to the regulation of collagen type I synthesis and degradation in their primary skin fibroblasts. To investigate this hypothesis, we analyzed collagen type I regulation in response to ionizing radiation and/or TGF-β1 stimuli, through quantitative assessment of the effectors potentially involved in collagen type I synthesis (TGF-β1 and VEGF), and its degradation (MMP-1, its inhibitor TIMP-1, and cathepsin K). In this prospective study, we also investigated possible correlations between collagen type I levels in primary fibroblasts derived from skin biopsies of early stage breast cancer patients and the incidence of acute skin toxicities in the same patients following their treatment with RT.

Chapter 2. Patients and Methods

2.1 Description of the study population

Patients were enrolled in an Institutional Review Board–approved protocol for a Phase III randomized controlled trial comparing three-dimensional conformal radiation therapy (3D-CRT) and Helical Tomotherapy (HT) in newly diagnosed women with breast cancer (ClinicalTrials.gov identifier: NCT00563407). All patients were recruited in a single institution at the Cross Cancer Institute. Eligible patients were ≥18 years with histologically proven diagnosis of invasive carcinoma of the breast (carcinoma in situ, Tumor 1-2, Node 0-1). The exclusion criteria included pregnancy, lactation, metastatic disease or collagen vascular disease. Written informed consent was obtained from each patient for the use of their skin biopsies, blood samples and clinical information. Patients were offered a guideline-based staging, surgery, adjuvant chemotherapy and RT. Patients were treated with adjuvant RT after lumpectomy. The dose delivered to the entire breast was 50 Gy in 25 fractions over 5 weeks. Acute and late skin toxicities are the primary endpoints of this clinical study. Acute skin toxicity was evaluated 6 to 8 weeks following RT using the National Cancer Institute's Common Toxicity Criteria, CTCAE v3: Common Toxicity Criteria Adverse Effects, Version 3.0. Low toxicity is defined as grade 0-1 and high toxicity as grade ≥2.

2.2 Derivation of primary human skin fibroblast cultures

Skin biopsies were excised from the forearm of patients prior to initiation of their RT treatment. Biopsies were sectioned into 8-10 small fragments under

sterile tissue culture conditions using scalpels. Each biopsy fragment was placed onto 35-mm polystyrene tissue culture plates and allowed to adhere for 10 minutes prior to addition of full fibroblast medium [DMEM-F12 media supplemented with 20% FBS, 1% antibiotic-antimycotic solution (Sigma Aldrich), 1% L-glutamine, and 1% amphotericin B solution (Sigma Aldrich)]. Human skin biopsy fragments remained in culture (14-21 days) until the appearance of outgrowths of spindle-shaped fibroblast cells. Fibroblasts were then treated with trypsin solution [0.017% EDTA and 0.25% trypsin (Gibco Bioproduction) in phosphate buffered saline (PBS)] for 10 minutes at 37°C and then transferred to 100-mm cell culture plates with 10 mL of full fibroblast medium for cell culture. Early passage fibroblasts were frozen for storage.

2.3 Cell culture

Primary human skin biopsy-derived fibroblasts were cultured and passaged in full fibroblast medium and incubated at 37°C and 5% CO_2. At each passage, fibroblasts at 90% confluency were trypsinized and split into 100-mm cell culture dishes at a 1:2 ratio. Fibroblasts were passaged to a maximum of passage 10.

GM-10 cells, a fetal human skin fibroblast cell line, and HL-60, a human promyelocytic leukemia cell line (kindly provided by Dr. Razmik Mirzayans) were cultured in DMEM-F12 media or DMEM media respectively, supplemented with 10% FBS, 1% antibiotic-antimycotic solution, 1% L-glutamine, and 1% amphotericin B solution.

2.4 *In vitro* cell culture treatment

Human skin fibroblasts at 90% confluence up to passage 8 were trypsinized, plated in 35-mm cell culture plates, and incubated at 37°C and 5% CO_2 in full fibroblast medium for 24 h to allow for surface adherence. Media was then removed and fibroblasts were gently rinsed with PBS to remove excess full fibroblast medium before addition of fibroblast starving medium (DMEM supplemented with 1% FBS, 1% antibiotic-antimyocotic solution, 1% L-glutamine, and 1% amphotericin B solution). Fibroblasts were cultured in fibroblast starving medium for 24 h and then medium was removed, followed by gentle washing with PBS. The fibroblasts were then treated or not treated with human TGF-β1-supplemented (1 ng/mL, Miltenyi Biotec) fibroblast starving medium, ionizing radiation (2 Gy, gamma source), or TGF-β1-supplemented fibroblast starving medium followed by ionizing radiation. TGF-β1 treatment was applied as a one-time bolus dose supplementing culture medium. Following treatment, fibroblasts were cultured for 24 h or 72 h in the same medium as described above.

2.5 Quantitative determination of collagen type I by ELISA

Primary human skin fibroblasts between passages 6-8 in 100-mm plates following a 72 h period after control treatment or treatment with TGF-β1 and/or ionizing radiation were treated with 0.05 M acetic acid (pH 2.8) after removal of culture supernatant. A cell scraper was used to lift the fibroblast cell layer from the surface of the tissue culture plate and transferred into 1 mL microcentrifuge tubes. Pepsin solution (10 mg/mL pepsin dissolved in 0.5 M acetic acid) was

added to the cell pellet and incubated for 24 h at 4°C with gentle mixing. 10x TBS (1 M Tris, 2 M NaCl, 50 mM $CaCl_2$, pH 7.8) was then added to the cell sample followed by neutralizing the sample to pH 8.0 with 1 N NaOH. Elastase solution (1 mg/mL elastase dissolved in TBS pH 7.8) was added, followed by 48 h incubation at 4°C with gentle mixing. Pepsin and elastase digested fibroblast cell sample was then centrifuged at 12,740xg for 5 minutes at room temperature. The resulting sample contained intracellular collagen type I as well as acid-solubilized secreted collagen type I. Seventy-two hour collagen type I cell extracts (supernatant) were collected and stored at -80°C. Twenty-four hour collagen type I cell extracts were not assayed after preliminary experiments showed insufficiently low collagen type I concentrations in 24 h cell extracts. Total protein concentration of collagen type I extracts was determined by the bicinchroninic acid assay (Thermo Scientific) and used to normalize collagen type I values between patients.

Human skin fibroblast collagen levels were measured by competitive ELISA using the Human Collagen Type I ELISA kit (MDBioproducts). Preliminary experiments were conducted to determine optimal dilution of samples. Samples, diluted 1:15 in assay diluent (buffer containing sodium azide), were thoroughly mixed with conjugate solution (containing biotinylated anti-collagen type I antibody) at working concentration and added to a collagen type I pre-coated microplate in duplicate. The microplate was sealed and incubated at room temperature with gentle mixing for 2 h, to allow competitive binding of the antibody between collagen type I in the sample and collagen type I on the coated

microplate. Following incubation, samples were removed from the microplate and washed with wash buffer (3 times, provided in kit). Streptavidin-HRP (provided in kit) diluted to working concentration was added to the microplate and incubated for 30 minutes at room temperature with gentle mixing. Streptavidin-HRP was then removed and washed with wash buffer (3 times). TMB substrate solution (provided by kit) was added to the microplate and incubated for 10 minutes at room temperature in the dark. Stop solution (1 N H_2SO_4) was then added to the microplate followed by gentle mixing. Sample optical density was read at 450 nm using an automated plate reader (Thermo Electric Multiskan Spectrum). Collagen type I concentrations were determined using a standard curve created with control values in an analysis program (SkanIt RE for MSS 2.4.2). After raw values were normalized between patient-derived samples using total protein concentration, data normalization between microplates was achieved using GM-10 collagen type I values. HL-60 was used as a collagen type I negative internal control within each microplate.

2.6 Quantitative determination of secreted factors

Cell culture supernatant samples were collected from primary human skin fibroblast cultures 24 h and 72 h after treatment, and centrifuged at 300xg for 10 minutes. Supernatant was collected, aliquoted into 0.5-mL microcentrifuge tubes, and stored at -20°C. Cell counting was conducted at 24 h and 72 h after treatment using an automated cell counter (Beckman Coulter Z2 Coulter Counter Analyzer). Fibroblast cell counts were used to normalize secreted factor values.

Secreted levels of TGF-β1, VEGF, MMP-1, or TIMP-1 in fibroblast culture supernatant were determined using their respective standard sandwich enzyme-linked immunosorbent assay (ELISA) Duoset kits (R&D Systems). 96-well ELISA microplates were treated with working concentrations of capture antibody overnight at room temperature. Excess capture antibody was then removed by washing with 0.05% Tween-20 in PBS (3 times).

TGF-β1, VEGF, MMP-1 or TIMP-1 concentrations were determined using a standard curve (analysis program: SkanIt RE for MSS 2.4.2). Preliminary experiments were conducted to determine optimal dilution of cell culture supernatants for each secreted factor. Based on those results, TGF-β1 and VEGF ELISAs were conducted using undiluted supernatants, while MMP-1 and TIMP-1 ELISAs were diluted (1:100) for optimal results.

For TGF-β1 ELISA, supernatants were incubating in 1 N HCl for 10 minutes at room temperature to activate all latent TGF-β1, followed by neutralization with 1.2 N NaOH/0.5 M HEPES. Activated supernatant was then immediately added to the microplate in duplicate.

Supernatant samples were added to the microplate and incubated at room temperature for 2 h. Unbound samples were then removed by washing with 0.05% Tween-20 in PBS. Detection antibody at working concentrations was added to the microplates and incubated at room temperature for 2 h, followed by removal of antibody and washing of microplates. Streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP, provided in kit) was used at a working dilution of 1:200 in 1% BSA in PBS and added to the microplates, followed by a

20 minute incubation at room temperature in the dark. Streptavidin-HRP was then removed and the microplate was washed with 0.05% Tween-20 in PBS (3 times). Trimethylbenzidine (TMB) solution (Sigma Aldrich) was added and incubated for 20 minutes at room temperature in the dark. Stop solution (2 N H_2SO_4) was then added, followed by reading of optical density using an automated microplate reader (Thermo Electric Multiskan Spectrum) at 450 nm. For each secreted factor, to normalize data between different microplates, samples of GM-10 supernatant cell culture were also processed and analyzed in parallel with samples from patients' fibroblasts.

2.7 Western blotting

2.7.1 Extraction of total cell lysates

Following removal and collection of cell culture supernatant, control and treated fibroblasts (24 h and 72 h) were washed with PBS, trypsinized (10 minutes at 37°C) then collected into a 15-mL tube by gentle flushing and pipetting using fibroblast starving media. After centrifugation (10 minutes at 300xg), the pellet was washed with ice-cold PBS, centrifuged (10 minutes at 300xg), then lysed using 200 µL of modified radio-immunoprecipitation assay (RIPA) cell lysis buffer (65 mM Tris base, 150 mM NaCl, 16 mM NP-40, 6 mM EDTA, pH 7.4) supplemented 30 minutes before use with 1% 0.1 M NaF, 0.01% protease inhibitor cocktail (Sigma Aldrich), and 0.5% $NaVO_4$. Lysates were transferred into a 1-mL microcentrifuge tube and spun for 10 minutes at 12,740xg at 4°C. Supernatant was collected, aliquoted, and stored at -80°C. Protein concentration

was measured with the bicinchroninic acid assay (Thermo Scientific) to determine loading volumes.

2.7.2 Gel electrophoresis

Cell lysate samples were combined with 4x sodium dodecyl sulphate (SDS) sample buffer (EMD Chemicals), boiled for 5 minutes at 95°C and the equivalent of 30 µg of protein were loaded per lane. GM-10 cell lysate was loaded as a positive control for active cathepsin K and HL-60 cell lysate was loaded as a negative control for active cathepsin K. To enable electrophoretic separation of cathepsin K, we used 9% SDS-PAGE separating gel [5 mL of 0.5 M Tris solution in H_2O pH 6.8, added to 6 mL of 30% acrylamide/bis-acrylamide (37.5:1, Bio-Rad) solution, 8.8 mL of H_2O, 150 µL of 10% ammonia persulfate, and 15 µL of tetramethylethylenediamine (TEMED, Invitrogen)] and 5% stacking gel [2.5 mL of 1.5 M Tris solution in H_2O pH 9.1, added to 1.75 mL of 30% acrylamide/bis-acrylamide (37.5:1) solution, 6 mL of H_2O, 200 µL of 10% ammonia persulfate, and 20 µL of TEMED]. Gels were cast in a BioRad mini-gel system. The SDS-PAGE was run in an electrophoretic gel apparatus with running buffer (0.15% Tris base, 0.72% glycine, 0.05% SDS, pH 8.3) at constant amperage of 20 mA.

2.7.3 Immunoblotting and development

Proteins were transferred to polyvinylidene fluoride (PVDF) membranes using a transferring sandwich setup. PVDF membrane was activated in methanol for 10 seconds then hydrated in H_2O for 5 minutes. Membrane, gels, filters, and sponges were equilibrated in transfer buffer (1.8% glycine, 0.38% Tris base, 20% methanol, 0.02% SDS) for 10 minutes. Transfer occurred at constant voltage of

120 V for 90 minutes at 4°C. After transfer, PVDF membrane was washed with TBS and blocked in 5% non-fat milk dissolved in TBS containing 0.1% Tween-20 (TBST) overnight. Membrane was then washed in TBST (three times, total of 30 minutes). Cathepsin K was detected using a rabbit polyclonal cathepsin K primary antibody (Abcam, 1:2000 dilution in 5% non-fat milk in TBST) incubated overnight at 4°C with gentle shaking. A goat anti-rabbit HRP conjugated secondary antibody (1:5000 dilution in 5% non-fat milk in TBST, Cell Signalling) was then applied and incubated for 1 h at room temperature. To ensure equal loading, we used anti-β actin antibody as a control. β-actin was detected following membrane stripping by incubation for 5 minutes at 37°C in stripping buffer (Thermo Scientific) using a mouse monoclonal primary antibody (1:10,000 dilution in 5% non-fat milk in TBST, Sigma) incubated for 30 minutes, followed by 1 h incubation with a goat anti-mouse HRP-conjugated secondary antibody (1:5000 dilution in 5% non-fat milk in TBST, Santa Cruz). ECL detection reagent was used as described by the manufacturer (Amersham Biosciences). Blots were exposed to Fuji Film Super RX medical X-ray film (using a Kodak X-OMAT 2000A film processor). After scanning exposed films, image contrast and brightness levels and densitometry analysis was conducted using Adobe Photoshop CS version 8.0 software.

2.8 Statistics

The Signed Rank test was used to calculate *P*. A *P* of <0.05 indicates significant differences between factor production/secretion values. The Signed Rank test allows non-parametric statistical analysis of differences between paired

values. Correlations between factor secretion and collagen type I production were assessed by calculating the non-parametric Spearman's rank correlation coefficient. Incidence of acute toxicity indicators of CTC grade, moist desquamation, and erythema were compared between primary fibroblasts separated into high (<1.42 units) and low (>1.42 units) collagen type I producing groups using the Pearson chi-squared test.

Chapter 3. Results

3.1 TGF-β1 significantly increased collagen type I levels in primary human skin fibroblasts

Fibroblasts contribute to ECM remodelling in radiation-induced injury through deposition of collagen type I. Exposure to IR and TGF-β1 secreted from mesenchymal cell types and cells involved in the injury response may significantly alter collagen type I levels in irradiated tissue. We quantitatively assessed collagen type I levels (intracellular and deposited collagen type I) by competitive ELISA for 40 patient-derived skin fibroblast samples untreated (control) or 72 h post treatment using TGF-β1 alone (1 ng/ml), IR alone (2 Gy), or TGF-β1 and IR combined treatment. Median baseline collagen type I production was 1.18 pg/mL/µg total protein (range = 0.19-2.62 pg/mL/µg total protein, n = 40, Table 1). Treatment with TGF-β1 significantly increased median collagen type I production (1.39 pg/mL/µg total protein, range = 0.36-3.87 pg/mL/µg total protein, P = 0.006). IR treatment alone did not significantly change collagen type I levels (median = 1.30 pg/mL/µg total protein, range = 0.16-2.59 pg/mL/µg total protein, P = 0.138). Combined TGF-β1 and IR treatment significantly increased collagen type I levels (median = 1.36 pg/mL/µg total protein, range = 0.21-3.46 pg/mL/µg total protein, P = 0.014, Table 1). Figure 4 shows a large inter-individual variation of collagen type I production at baseline levels and following each type of treatment. We sub-grouped the population of 40 patient-derived fibroblast samples into low (median = 0.77 pg/mL/µg total protein, range = 0.19-0.92 pg/mL/µg total protein, n = 13), mid (median = 1.18 pg/mL/µg total protein,

range = 0.96-1.40 pg/mL/μg total protein, n = 14), and high (median = 1.66 pg/mL/μg total protein, range = 1.45-2.62 pg/mL/μg total protein, n = 13) collagen type I-secreting groups based on baseline collagen type I levels (Fig. 5). The fibroblasts of patients secreting high basal levels of collagen type I were prone to produce high levels following TGF-β1 treatment or the combined treatment of TGF-β1 and IR. We observed the same trend in both the mid and low sub-populations. Thus, baseline levels seem to pre-determine the range of collagen type I levels following each type of treatment.

	Baseline	TGF-β1 treatment (1ng/mL)		IR treatment (2Gy)		TGF-β1 and IR combined	
	Median (Range)†	Median (Range)†	P-value	Median (Range)†	P-value	Median (Range)†	P-value
72h	1.18 (0.19-2.62)	1.39 (0.36-3.87)	0.006**	1.30 (0.16-2.59)	0.138	1.36 (0.21-3.46)	0.014*

Table 1. Primary fibroblast collagen type I levels (n = 40). Patient-derived fibroblasts were treated or not (baseline) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 72 h. Collagen type I levels in primary fibroblast culture samples determined by ELISA. Values normalized to total protein concentration of samples. †median and range values shown in pg/mL/μg total protein. TGF-β1=transforming growth factor-β1, IR=ionizing radiation, *P* calculated with Signed Ranks test using difference compared to baseline.

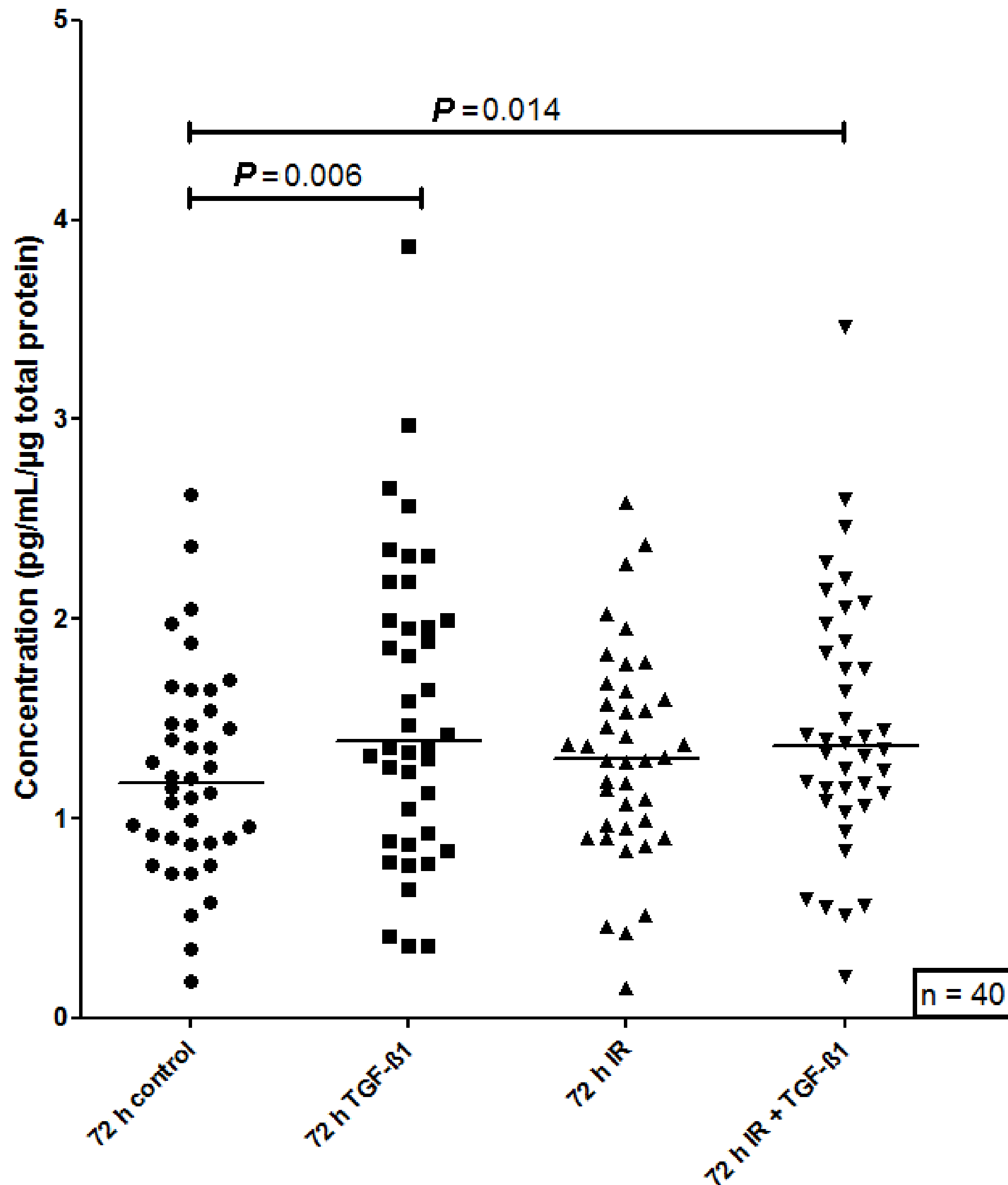
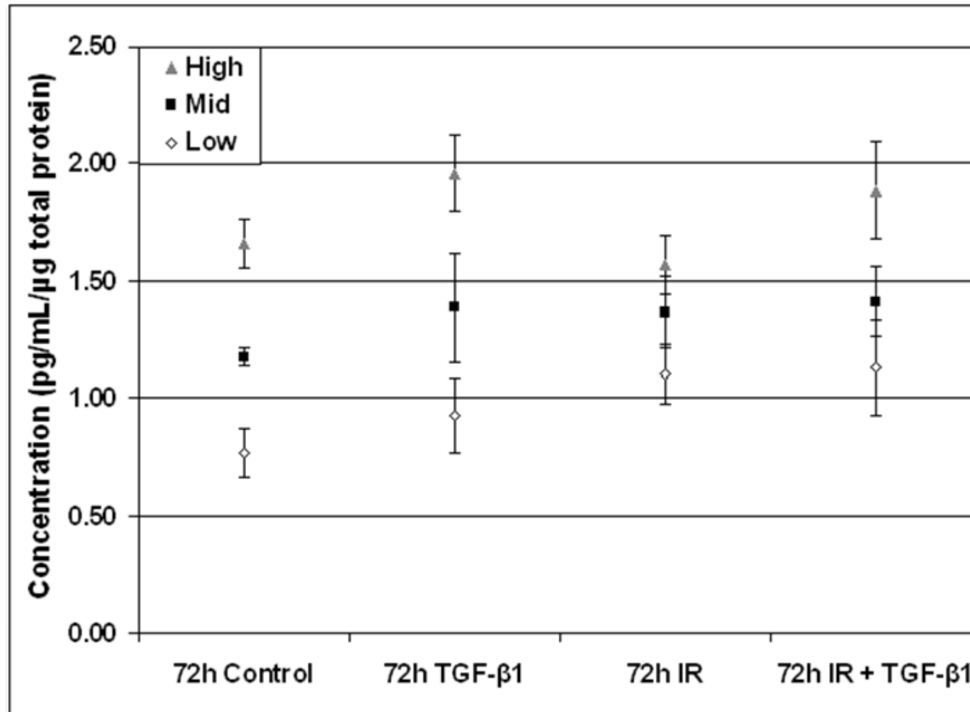


Figure 4. Primary fibroblast collagen type I levels increase following TGF-β1 and IR treatment (n = 40). Primary human skin fibroblasts starved for 24 h were treated or not (control) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 72 h. Collagen type I levels in the culture supernatant were measured by ELISA at 72 h. Bars denote median collagen type I levels. TGF-β1=transforming growth factor-β1, IR=ionizing radiation, *P* calculated with Signed Ranks test using difference compared to baseline.



High	Median	1.66	1.96	1.57	1.89
	min	1.45	0.88	0.97	0.51
	max	2.62	2.97	2.37	3.46
Mid	Median	1.18	1.39	1.37	1.41
	min	0.96	0.41	0.42	0.59
	max	1.40	3.87	2.59	2.60
Low	Median	0.77	0.93	1.10	1.13
	min	0.19	0.36	0.16	0.21
	max	0.92	2.57	1.82	1.83

Figure 5. Behavior of low, mid, and high 72h baseline collagen type I expressing primary fibroblast populations. Using baseline collagen type I levels as determined by ELISA, 40 patient-derived fibroblast samples were separated into 3 groups: low (n = 13), mid (n = 14), and high (n = 15). Median collagen type I response of each group to treatments are shown as above. Values shown in pg/mL/µg total protein. Error bars based on standard error. TGF-β1=transforming growth factor-β1, IR=ionizing radiation.

3.2 TGF-β1 alone and combined with IR significantly increased secretion of TGF-β1 in primary human skin fibroblasts

TGF-β1 is a potent inducer of fibroblast collagen type I synthesis [62], thus the modulation of TGF-β1 levels by irradiation in a TGF-β1-rich environment may prove to be of importance in regulating collagen type I levels in skin fibroblasts during radiation injury. Table 2 shows quantitative analysis of active TGF-β1 by ELISA in 22 primary fibroblast cultures. Median fibroblast baseline secretion of TGF-β1 at 24 h was 20.29 pg/mL/10^5 cells (range = 0.94-127.62 pg/mL/10^5 cells, n = 22, Table 2). At 24 h after treatment with TGF-β1 (1 ng/mL), primary fibroblast TGF-β1 secretion slightly but significantly increased (median = 24.65 pg/ml/10^5 cells, range = 8.70-138.17 pg/ml/10^5 cells, $P = 0.009$). Although, this increase was very slight and while treatment with IR (2 Gy) did not significantly alter TGF-β1 secretion, the combined treatment of TGF-β1 and IR induced increased TGF-β1 levels (median = 29.43 pg/ml/10^5 cells, range = 4.28-168.99 pg/ml/10^5 cells, $P < 0.001$). Baseline primary fibroblast TGF-β1 secretion at 72 h (46.02 pg/mL/10^5 cells, range = 6.02-154.36 pg/mL/10^5 cells) increased 2-fold compared to baseline TGF-β1 secretion at 24 h. Following IR treatment at 72 h, TGF-β1 secretion significantly increased (median = 53.21 pg/ml/10^5 cells, range = 10.23-251.41 pg/ml/10^5 cells, $P < 0.001$). The combined treatment of TGF-β1 (1 ng/mL) and IR (2 Gy) yielded a significant increase in median TGF-β1 secretion at 72 h (median = 57.47 pg/ml/10^5 cells, range = 18.55-199.55 pg/ml/10^5 cells, $P < 0.001$) (Table 2). Figure 6 illustrates the considerable variability observed between patient-derived fibroblast TGF-β1 secretions.

Separation of the patient-derived fibroblast population baseline TGF-β1 secretion at 24 h allowed the allocation of patient-derived fibroblast cultures into the following TGF-β1 secreting groups: high (median = 67.47 pg/ml/10^5 cells, range = 49.47-127.62 pg/ml/10^5 cells, n = 7), mid (median = 20.29 pg/ml/10^5 cells, range = 15.26-47.39 pg/ml/10^5 cells, n = 8), and low (median = 8.87 pg/ml/10^5 cells, range = 0.94-15.11 pg/ml/10^5 cells, n = 7) (Fig. 7). Median levels of TGF-β1 secretion in response to treatment remained within the same high, mid, or low TGF-β1 secreting groups, suggesting distinct responses within each group as determined by baseline fibroblast TGF-β1 secretion. In summary, our results suggest that TGF-β1 exerts an autocrine effect at 24 h, as secretion of TGF-β1 by primary skin fibroblasts was significantly increased by exogenous TGF-β1 stimulation. Increased TGF-β1 secretion following IR treatment 72 h post treatment was further enhanced when IR was combined to TGF-β1 stimuli. This could be of great significance with respect to the subsequent effects of secreted TGF-β1 as an autocrine factor with a central role in collagen type I regulation.

	Baseline	TGF-β1 treatment (1 ng/mL)		IR treatment (2 Gy)		TGF-β1 and IR combined	
	Median (Range)†	**Median (Range)†**	***P***	**Median (Range)†**	***P***	**Median (Range)†**	***P***
24 h	20.29 (0.94-127.62)	24.65 (8.70-138.17)	0.009 **	26.96 (1.74-166.18)	0.163	29.43 (4.28-168.99)	<0.001 ***
72 h	46.02 (6.02-154.36)	40.36 (11.11-161.82)	0.695	53.21 (10.23-251.41)	<0.001 ***	57.47 (18.55-199.55)	<0.001 ***

Table 2. TGF-β1 and IR combined treatment increases human skin fibroblast TGF-β1 secretion (n = 22). Fibroblasts were treated or not (baseline) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 24h or 72 h. Secreted TGF-β1 in the culture supernatant was determined by ELISA. †pg/mL/10^5 cells. TGF-β1=transforming growth factor-β1, IR=ionizing radiation, *P* calculated with Signed Ranks test using difference compared to control.

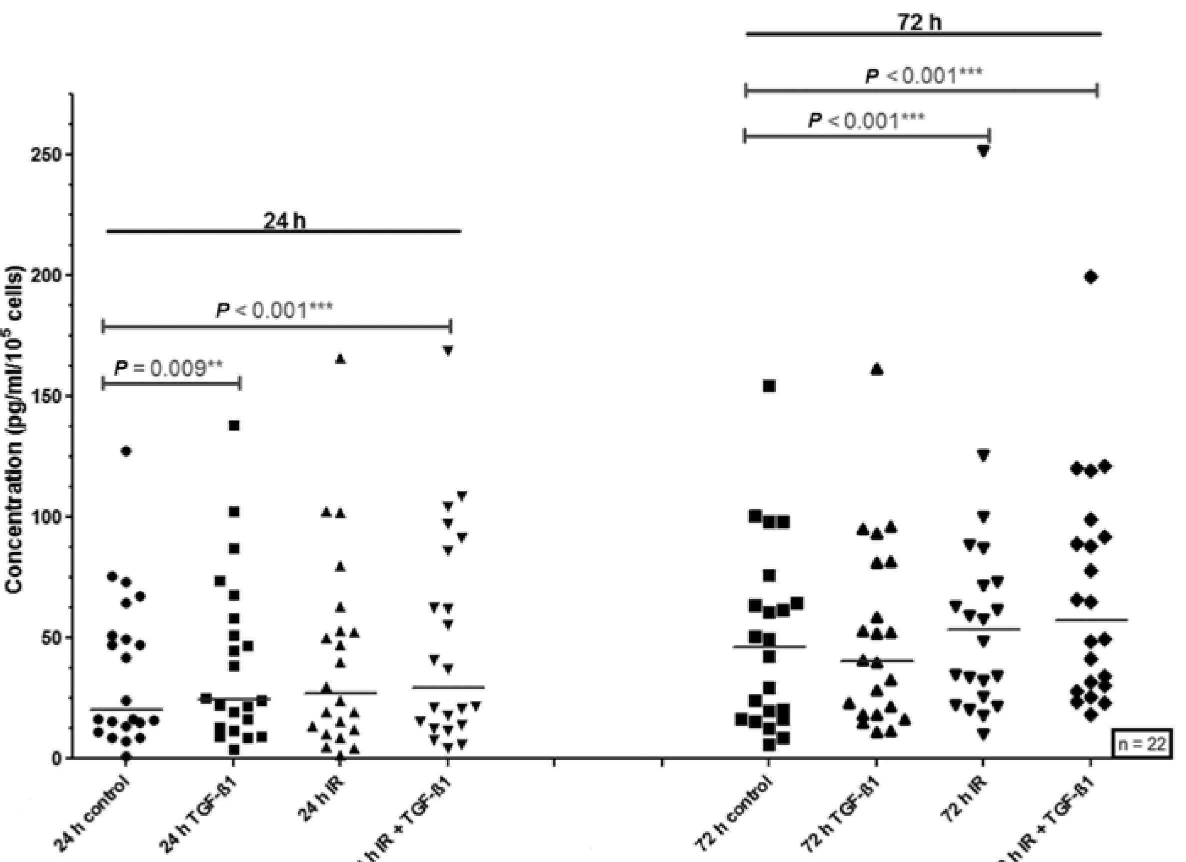
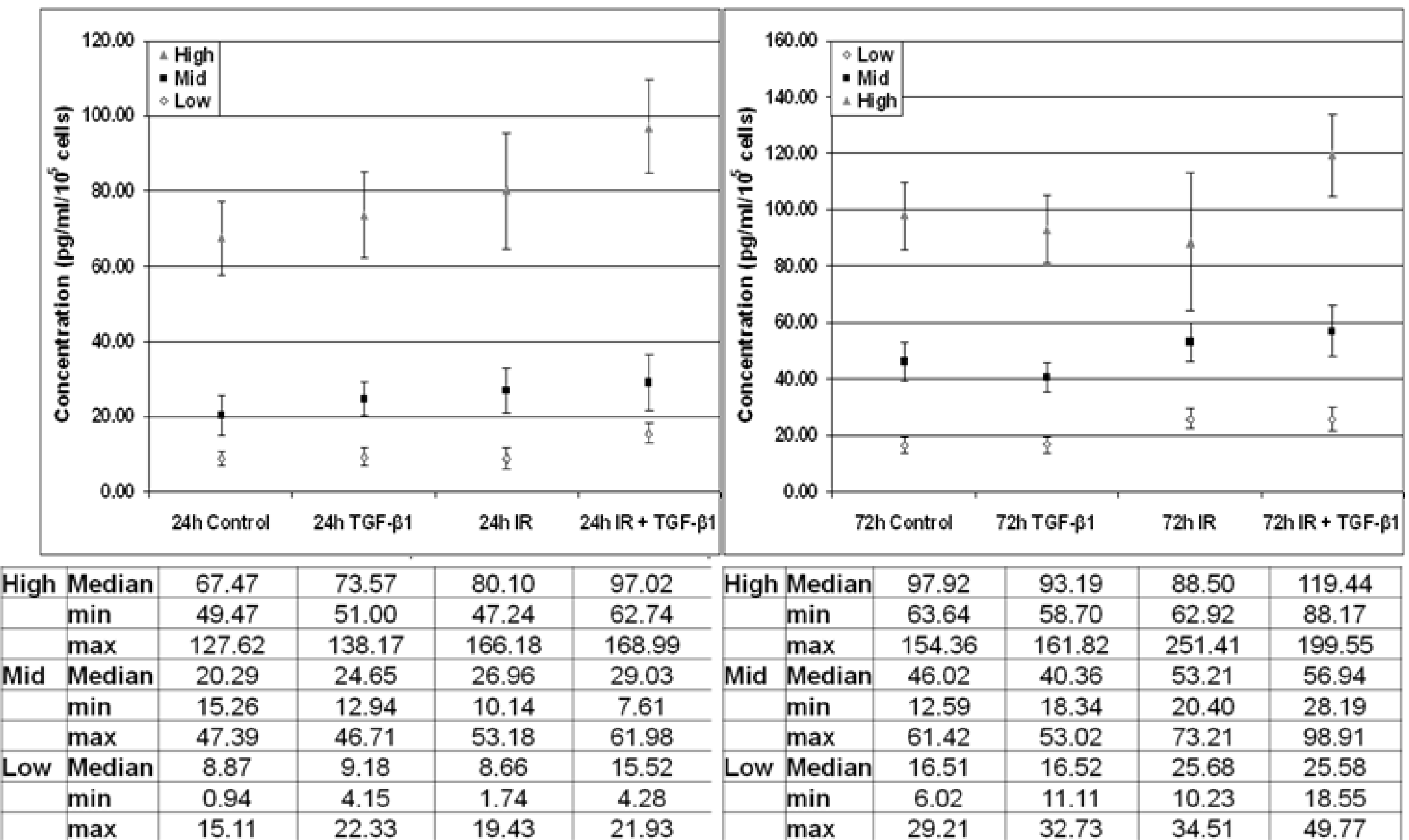


Figure 6. Primary fibroblast TGF-β1 secretion increases after TGF-β1 and IR treatment (n = 22). Primary human skin fibroblasts starved for 24 h were treated or not (control) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 24 h or 72 h. TGF-β1 secretion was determined by ELISA in culture supernatant. Bars denote median TGF-β1 secretion. TGF-β1=transforming growth factor-β1, IR=ionizing radiation, P calculated with Signed Ranks test using difference compared to baseline.



		24h Control	24h TGF-β1	24h IR	24h IR + TGF-β1
High	Median	67.47	73.57	80.10	97.02
	min	49.47	51.00	47.24	62.74
	max	127.62	138.17	166.18	168.99
Mid	Median	20.29	24.65	26.96	29.03
	min	15.26	12.94	10.14	7.61
	max	47.39	46.71	53.18	61.98
Low	Median	8.87	9.18	8.66	15.52
	min	0.94	4.15	1.74	4.28
	max	15.11	22.33	19.43	21.93

		72h Control	72h TGF-β1	72h IR	72h IR + TGF-β1
High	Median	97.92	93.19	88.50	119.44
	min	63.64	58.70	62.92	88.17
	max	154.36	161.82	251.41	199.55
Mid	Median	46.02	40.36	53.21	56.94
	min	12.59	18.34	20.40	28.19
	max	61.42	53.02	73.21	98.91
Low	Median	16.51	16.52	25.68	25.58
	min	6.02	11.11	10.23	18.55
	max	29.21	32.73	34.51	49.77

Figure 7. Behavior of low, mid, and high baseline TGF-β1 secreting primary fibroblast populations. Using baseline TGF-β1 secretion levels at 24 h under control condition as determined by ELISA, 22 patient-derived fibroblast samples were separated into 3 groups: low (n = 7), mid (n = 8), and high (n = 7). Median TGF-β1 secretion response of each group to treatments at 24 h and 72 h after treatment are shown as above. Values shown in pg/mL/10^5 cells. Error bars based on standard error. TGF-β1=transforming growth factor-β1, IR=ionizing radiation.

3.3 TGF-β1 alone and combined with IR significantly increased secretion of VEGF in primary human skin fibroblasts

Fibroblast secretion of VEGF as a consequence of radiation injury may play a role in promoting migration of cells involved in wound repair and collagen type I deposition. We assessed by ELISA whether VEGF secretion was modulated following different types of treatment compared to baseline levels at both 24 h and 72 h in a group of 22 patient-derived fibroblast cultures. At the early time point of 24 h, treatment with TGF-β1 alone induced a significant 2-fold increase in VEGF secretion (median = 123.91 pg/mL/10^5 cells, range = 19.51-417.08 pg/ml/10^5 cells, $P < 0.001$) compared to baseline (median = 67.90 pg/ml/10^5 cells, range = 8.50-125.35 pg/ml/10^5 cells, Table 3). IR treatment alone did not induce a significant change in median VEGF secretion. Combined treatment of TGF-β1 and IR increased secretion of VEGF by more that 2-fold within 24 h (median = 137.86 pg/ml/10^5 cells, range = 26.20-356.53 pg/ml/10^5 cells, $P < 0.001$). Median VEGF baseline secretion at 72 h was 77.34 pg/ml/10^5 cells (range = 17.11-249.99 pg/ml/10^5 cells). Treatment with TGF-β1 alone significantly increased VEGF secretion (median = 151.48 pg/ml/10^5 cells, range = 30.19-360.98 pg/ml/10^5 cells, $P < 0.001$) after 72 h. IR treatment induced a slight but significant increase in VEGF secretion after 72 h, with a median VEGF secretion of 87.84 pg/mL/10^5 (range = 3.71-257.81 pg/ml/10^5 cells, $P = 0.027$). Treatment with TGF-β1 and IR after 72 h induced a larger increase of VEGF compared to each treatment alone (median = 212.64 pg/ml/10^5 cells, range = 36.32-616.41 pg/ml/10^5 cells, $P < 0.001$, Table 3). Within the group of 22

patient-derived skin fibroblast samples, response to treatment was more pronounced for some outlier cases with much higher VEGF secretion compared to median values (Fig. 8). We separated patient-derived fibroblast samples into 3 VEGF-secreting groups using 24 h VEGF baseline secretion as follows: low (median = 10.82 pg/ml/10^5 cells, range = 8.50-39.43 pg/ml/10^5 cells, n = 7), mid (median = 67.90 pg/ml/10^5 cells, range = 44.64-79.01 pg/ml/10^5 cells, n = 8), and high (median = 100.26 pg/ml/10^5 cells, range = 86.80-125.35 pg/ml/10^5 cells, n = 7). The distinct profiles of VEGF secretion as observed in Figure 9 suggest that VEGF secretion in response to treatment may be dependent on baseline levels of VEGF. Our observations show that primary fibroblast VEGF secretion was increased by TGF-β1 stimulation. Though IR treatment alone induced little VEGF secretion, when combined with TGF-β1 treatment, primary fibroblast VEGF secretion was further enhanced.

	Baseline	TGF-β1 treatment (1 ng/mL)		IR treatment (2 Gy)		TGF-β1 and IR combined	
	Median (Range)†	Median (Range)†	*P*	Median (Range)†	*P*	Median (Range)†	*P*
24 h	67.90 (8.50-125.35)	123.91 (19.51-417.08)	<0.001 ***	57.58 (10.35-168.30)	0.318	137.86 (26.20-385.53)	<0.001 ***
72 h	77.34 (17.11-249.99)	151.48 (30.19-360.98)	<0.001 ***	87.84 (3.71-257.81)	0.027*	212.64 (36.32-616.41)	<0.001 ***

Table 3. TGF-β1 treatment alone and in combination with IR increased fibroblast VEGF secretion (n = 22). Fibroblasts were treated or not (baseline) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 24h or 72 h. Secreted VEGF in the culture supernatant was determined by ELISA. †pg/mL/10^5 cells. VEGF=vascular endothelial growth factor, TGF-β1=transforming growth factor-β1, IR=ionizing radiation, *P* calculated with Signed Ranks test using difference compared to control.

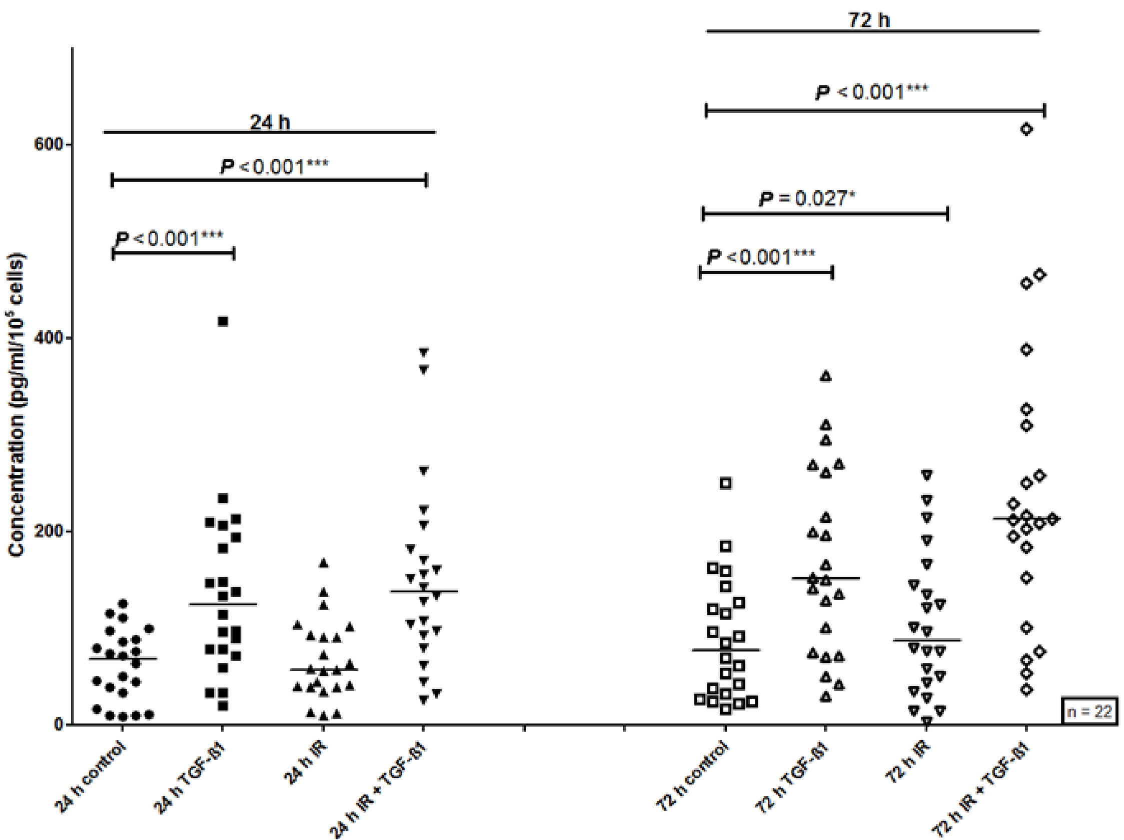
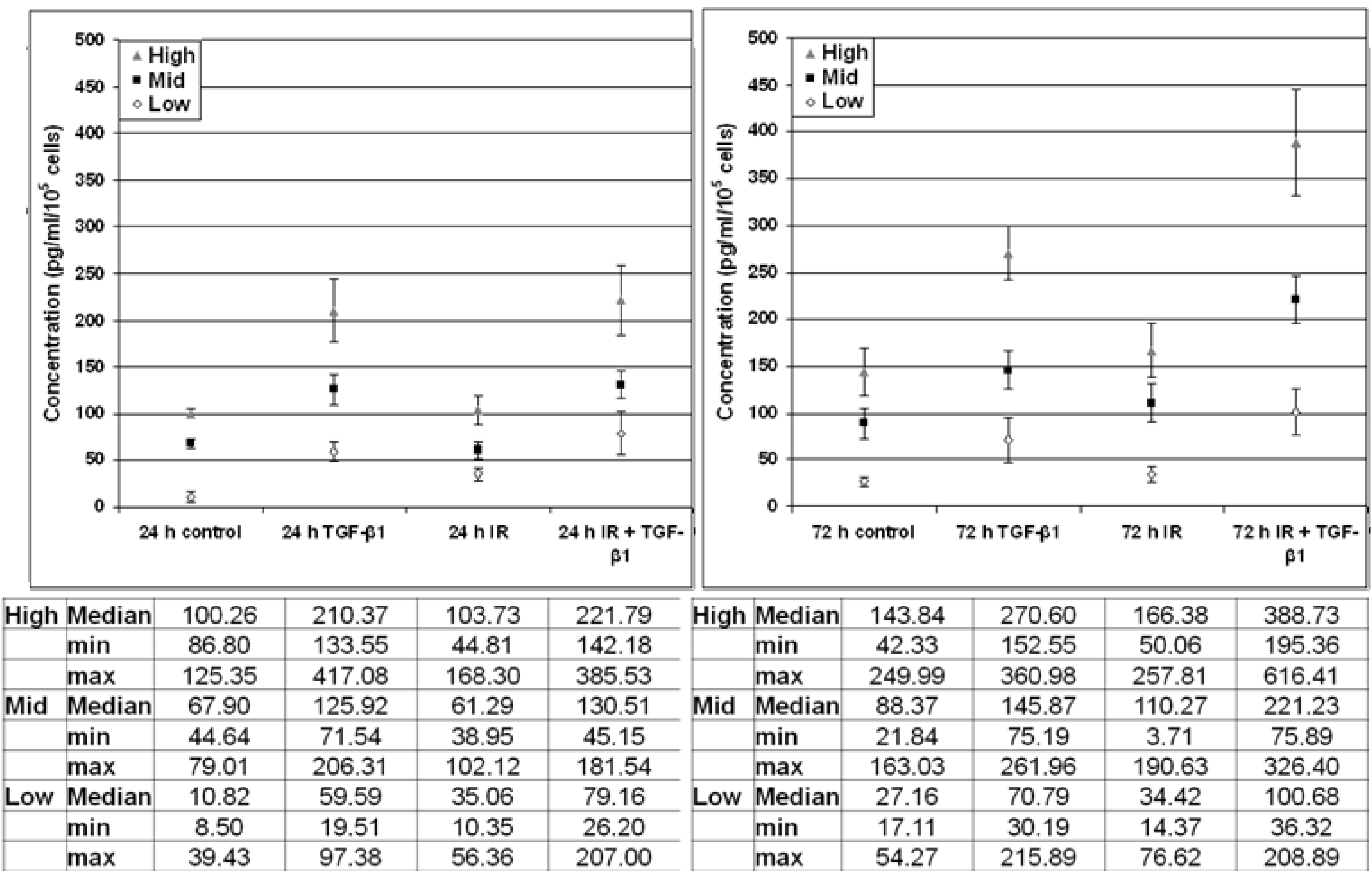


Figure 8. Primary fibroblast VEGF secretion increases with TGF-β1 treatment alone and TGF-β1 + IR treatment (n = 22). Primary human skin fibroblasts starved for 24 h were treated or not (control) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 24 h or 72 h. VEGF secretion was determined by ELISA in culture supernatant. Bars denote median VEGF secretion. TGF-β1=transforming growth factor-β1, VEGF=vascular endothelial growth factor, IR=ionizing radiation, *P* calculated with Signed Ranks test using difference compared to baseline.



		24 h control	24 h TGF-β1	24 h IR	24 h IR + TGF-β1
High	Median	100.26	210.37	103.73	221.79
	min	86.80	133.55	44.81	142.18
	max	125.35	417.08	168.30	385.53
Mid	Median	67.90	125.92	61.29	130.51
	min	44.64	71.54	38.95	45.15
	max	79.01	206.31	102.12	181.54
Low	Median	10.82	59.59	35.06	79.16
	min	8.50	19.51	10.35	26.20
	max	39.43	97.38	56.36	207.00

		72 h control	72 h TGF-β1	72 h IR	72 h IR + TGF-β1
High	Median	143.84	270.60	166.38	388.73
	min	42.33	152.55	50.06	195.36
	max	249.99	360.98	257.81	616.41
Mid	Median	88.37	145.87	110.27	221.23
	min	21.84	75.19	3.71	75.89
	max	163.03	261.96	190.63	326.40
Low	Median	27.16	70.79	34.42	100.68
	min	17.11	30.19	14.37	36.32
	max	54.27	215.89	76.62	208.89

Figure 9. Behavior of low, mid, and high baseline VEGF secreting primary fibroblast populations. Using baseline VEGF secretion levels at 24 h under control condition as determined by ELISA, 22 patient-derived fibroblast samples were separated into 3 groups: low (n = 7), mid (n = 8), and high (n = 7). Median VEGF secretion response of each group to treatments at 24 h and 72 h after treatment are shown as above. Values shown in pg/mL/10^5 cells. Error bars based on standard error. TGF-β1=transforming growth factor-β1, VEGF=vascular endothelial growth factor, IR=ionizing radiation.

3.4 TGF-β1 treatment significantly decreased MMP-1 secretion in primary human skin fibroblasts

Fibroblast deposition of collagen type I is counterbalanced by the collagenolytic activity of MMP-1. IR stimulation and increased levels of TGF-β1 in a radiation wound region can potentially change fibroblast secretion of MMP-1 and alter the balance between collagen type I deposition and degradation. We tested the same group of patient-derived skin fibroblast samples (n = 22) for MMP-1 secretion at baseline and following treatment with TGF-β1 and/or IR at 24 and 72 h. Table 4 shows that MMP-1 secretion was significantly decreased at 24 h by TGF-β1 treatment alone (median = 64116 pg/ml/10^5 cells, range = 25507-249604 pg/ml/10^5 cells, $P = 0.022$) compared to MMP-1 baseline secretion at 24 h (median = 67744 pg/ml/10^5 cells, range = 29213-578498 pg/ml/10^5 cells). Treatment with IR alone did not yield a statistically significant shift in MMP-1 secretion after 24 h, though we noted a slight trend towards increased MMP-1 secretion (median = 80232 pg/ml/10^5 cells, range = 27461-375770 pg/ml/10^5 cells, $P = 0.065$). The combined treatment did not significantly affect MMP-1 secretion at 24 h (median = 72920 pg/ml/10^5 cells, range = 31528-331671 pg/ml/10^5 cells, $P = 0.672$). At 72 h, MMP-1 secretion was once again significantly reduced after treatment with TGF-β1 alone (median = 63630 pg/ml/10^5 cells, range = 32926-405613 pg/ml/10^5 cells, $P < 0.001$) compared to baseline MMP-1 secretion (median = 96189 pg/ml/10^5 cells, range = 34586-797608 pg/ml/10^5 cells). The effect of IR treatment alone was more pronounced at 72 h, compared to 24 h, as MMP-1 secretion was nearly significantly increased (median = 100302 pg/ml/10^5

cells, range = 46680-3903986 pg/ml/10^5 cells, $P = 0.051$). Combined treatment with TGF-β1 and IR produced no significant change in median MMP-1 secretion after 72 h (88753 pg/ml/10^5 cells, range = 46372-436281 pg/ml/10^5 cells, $P = 0.334$, Table 4). As illustrated in Figure 10, the distribution of MMP-1 secretion included several outliers. Those outliers correspond to high MMP-1 secretion observed in specific patient-derived fibroblast samples that responded to treatment with high MMP-1 secretion. Using 24 h baseline fibroblast MMP-1 secretion, fibroblast samples were separated into low (median = 35462 pg/ml/10^5 cells, range = 29213-53408 pg/ml/10^5 cells, n = 7), mid (median = 67744 pg/ml/10^5 cells, range = 53408-104951 pg/ml/10^5 cells, n = 8), and high (median = 137357 pg/ml/10^5 cells, range = 119567-578498 pg/ml/10^5 cells, n = 7) MMP-1-secreting groups. As suggested in Figure 11, our group of patient-derived fibroblasts includes a sub-population of high MMP-1-secreting fibroblasts. In summary, our results show that within our group of 22 patient-derived primary fibroblast samples, median MMP-1 secretion was decreased by TGF-β1 treatment alone, but not significantly altered by IR treatment alone. This may have antagonized the effect of TGF-β1, as combined treatment with TGF-β1 and IR did not significantly affect levels of MMP-1 at the time points and doses tested in our experimental model. In this context, the effect of this treatment on TIMP-1 levels has to be investigated.

	Baseline	TGF-β1 treatment (1 ng/mL)		IR treatment (2 Gy)		TGF-β1 and IR combined	
	Median (Range)†	Median (Range)†	*P*	Median (Range)†	*P*	Median (Range)†	*P*
24 h	67744 (29213-578498)	64116 (25507-249604)	0.022*	80323 (27461-375770)	0.065	72920 (31528-331671)	0.672
72 h	96189 (34586-797608)	63630 (32926-405613)	<0.001 ***	100302 (46680-3903986)	0.051	88753 (46379-436281)	0.334

Table 4. TGF-β1 treatment alone significantly decreased fibroblast MMP-1 secretion (n = 22). Fibroblasts were treated or not (baseline) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 24h or 72 h. Secreted MMP-1 in culture supernatant was determined by ELISA. †pg/mL/10^5 cells. MMP-1=matrix metalloproteinase-1, TGF-β1=transforming growth factor-β1, IR=ionizing radiation, *P* calculated with Signed Ranks test using difference compared to control.

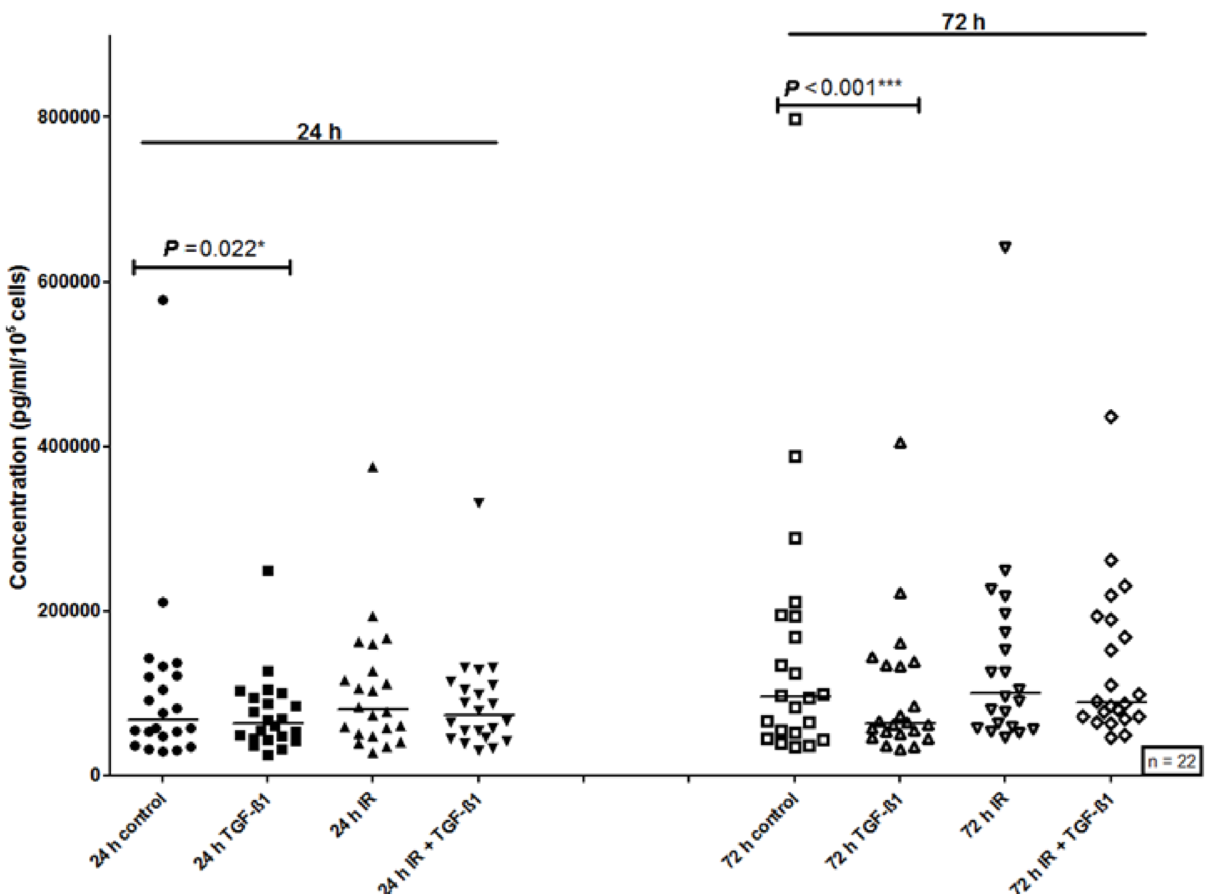
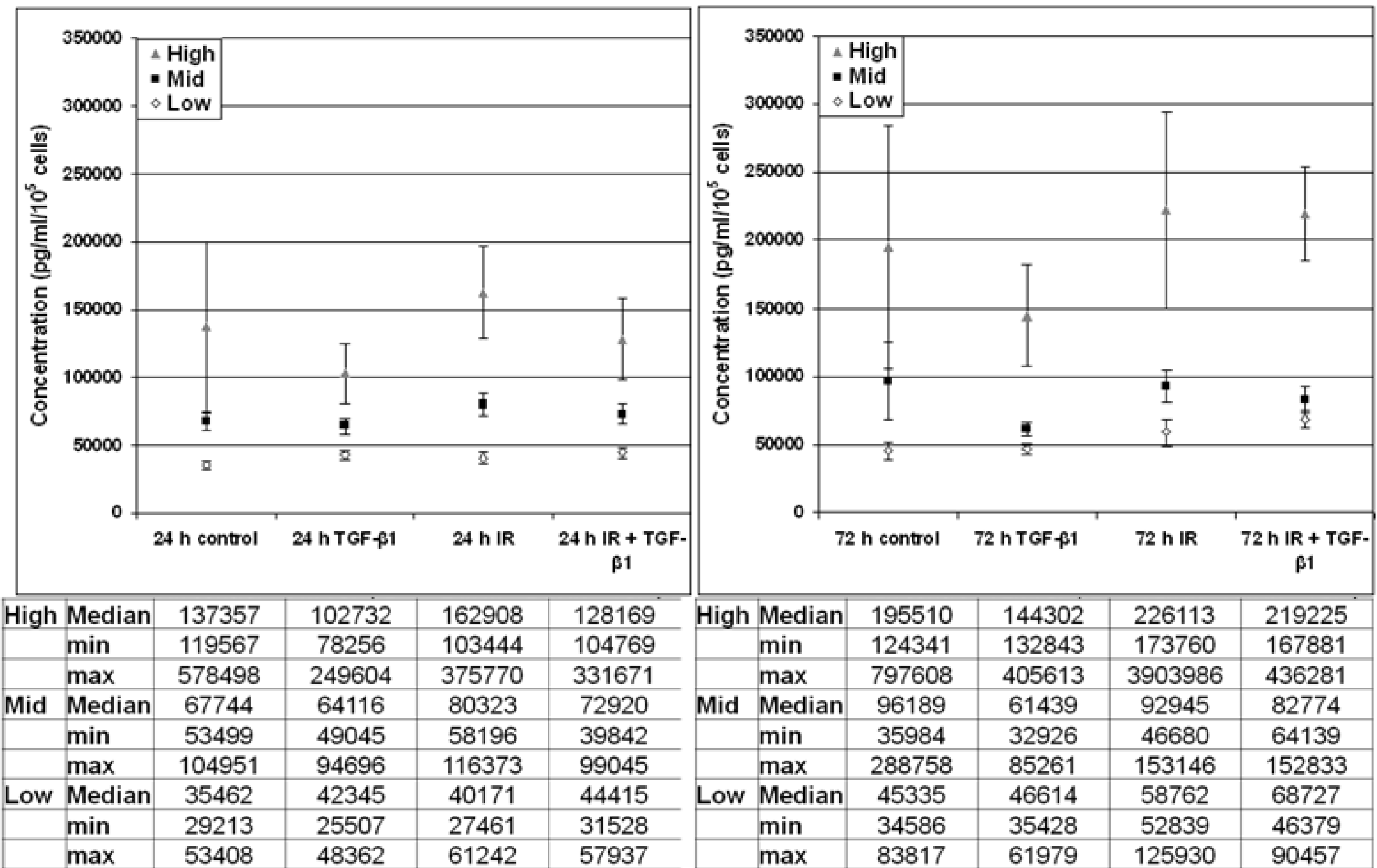


Figure 10. Primary fibroblast MMP-1 secretion increases with TGF-β1 treatment (n = 22). Primary human skin fibroblasts starved for 24 h were treated or not (control) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 24 h or 72 h. MMP-1 secretion was determined by ELISA in culture supernatant. Bars denote median MMP-1 secretion. MMP-1=matrix metalloproteinase-1, TGF-β1=transforming growth factor-β1, IR=ionizing radiation, P calculated with Signed Ranks test using difference compared to control.



		24 h control	24 h TGF-β1	24 h IR	24 h IR + TGF-β1
High	Median	137357	102732	162908	128169
	min	119567	78256	103444	104769
	max	578498	249604	375770	331671
Mid	Median	67744	64116	80323	72920
	min	53499	49045	58196	39842
	max	104951	94696	116373	99045
Low	Median	35462	42345	40171	44415
	min	29213	25507	27461	31528
	max	53408	48362	61242	57937

		72 h control	72 h TGF-β1	72 h IR	72 h IR + TGF-β1
High	Median	195510	144302	226113	219225
	min	124341	132843	173760	167881
	max	797608	405613	3903986	436281
Mid	Median	96189	61439	92945	82774
	min	35984	32926	46680	64139
	max	288758	85261	153146	152833
Low	Median	45335	46614	58762	68727
	min	34586	35428	52839	46379
	max	83817	61979	125930	90457

Figure 11. Behavior of low, mid, and high baseline MMP-1 secreting primary fibroblast populations. Using baseline MMP-1 secretion levels at 24 h under control condition as determined by ELISA, 22 patient-derived fibroblast samples were separated into 3 groups: low (n = 7), mid (n = 8), and high (n = 7). Median MMP-1 secretion response of each group to treatments at 24 h and 72 h after treatment are shown as above. Values shown in pg/mL/10^5 cells. Error bars based on standard error. TGF-β1=transforming growth factor-β1, MMP-1=matrix metalloproteinase-1, IR=ionizing radiation.

3.5 IR treatment alone and in combination with TGF-β1 induced fibroblast TIMP-1 secretion

The collagenolytic activity of MMP-1 is subject to inhibition by TIMP-1, which may play a role in collagen type I overproduction by fibroblasts during radiation injury. Determination of TIMP-1 secretion was conducted by ELISA for 22 patient-derived fibroblast samples. Median baseline TIMP-1 secretion at 24 h (25423 pg/ml/10^5 cells, range = 9315-73536 pg/ml/10^5 cells) increased by 2-fold at the later time point of 72 h (median = 46485 pg/ml/10^5 cells, range = 18063-166104 pg/ml/10^5 cells). Treatment with TGF-β1 alone did not induce significant changes in median TIMP-1 secretion compared to baseline levels at 24 h and 72 h (Table 5). IR treatment alone induced a slight, but significant increase in TIMP-1 secretion at both time points. Twenty-four hours after IR treatment, median TIMP-1 secretion increased (31072 pg/ml/10^5 cells, range = 11257-68151 pg/ml/10^5 cells, $P = 0.022$). At 72 h post IR treatment, median TIMP-1 secretion increased (49222 pg/ml/10^5 cells, range = 17574-114064 pg/ml/10^5 cells, $P = 0.020$). Treatment with TGF-β1 and IR combined also resulted in significant increases in TIMP-1 secretion. Following combined treatment, median TIMP-1 secretion increased (29906 pg/ml/10^5 cells, range = 13359-234878 pg/ml/10^5 cells, $P = 0.002$) compared to median baseline TIMP-1 secretion at 24 h. Combined TGF-β1 and IR treatment increased median TIMP-1 secretion after 72 h (58327 pg/ml/10^5 cells, range = 24917-172590 pg/ml/10^5 cells, $P < 0.001$) compared to median baseline TIMP-1 secretion at 72 h (46485 pg/ml/10^5 cells, range = 18063-166104 pg/ml/10^5 cells, Table 5). Figure 12 illustrates the significant increase in

TIMP-1 secretion induced by IR treatment alone and combined TGF-β1 and IR treatment, including the most substantial increase in TIMP-1 induced 72 h after combined TGF-β1 and IR treatment. Figure 13 shows that after separating the population based on 24h baseline TIMP-1 secretion in 3 groups: low (median = 13295 pg/ml/10^5 cells, range = 9315-16934 pg/ml/10^5 cells, n = 7), mid (median = 25423 pg/ml/10^5 cells, range = 21354-30649 pg/ml/10^5 cells, n = 8), high (median = 47753 pg/ml/10^5 cells, range = 37042-73536 pg/ml/10^5 cells, n = 7), these subpopulations once again tend to remain distinct in levels of TIMP-1 secretion based on baseline TIMP-1 secretion. These observations indicate that fibroblast TIMP-1 secretion was not altered by TGF-β1 stimulation, and responded to IR treatment with a slow increase. Combined TGF-β1 and IR treatment resulted in a greater increase in fibroblast TIMP-1 secretion, which may affect the balance of collagen type I synthesis and degradation.

	Baseline	TGF-β1 treatment (1 ng/mL)		IR treatment (2 Gy)		TGF-β1 and IR combined	
	Median (Range)†	Median (Range)†	*P*	Median (Range)†	*P*	Median (Range)†	*P*
24 h	25423 (9315-73536)	31072 (11257-68151)	0.173	26784 (11546-229611)	0.022*	29906 (13359-234878)	0.002 **
72 h	46485 (18063-166104)	49222 (17574-114064)	0.672	49222 (17454-151603)	0.020*	58327 (24917-172590)	<0.001 ***

Table 5. IR treatment alone and in combination with TGF-β1 induced fibroblast TIMP-1 secretion (n = 22). Fibroblasts were treated or not (baseline) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 24h or 72 h. Secreted TIMP-1 in primary fibroblast culture supernatant determined by ELISA. †pg/mL/10^5 cells. TIMP-1=tissue inhibitor of metalloproteinase-1, TGF-β1=transforming growth factor-β1, IR=ionizing radiation, *P* calculated with Signed Ranks test using difference compared to control.

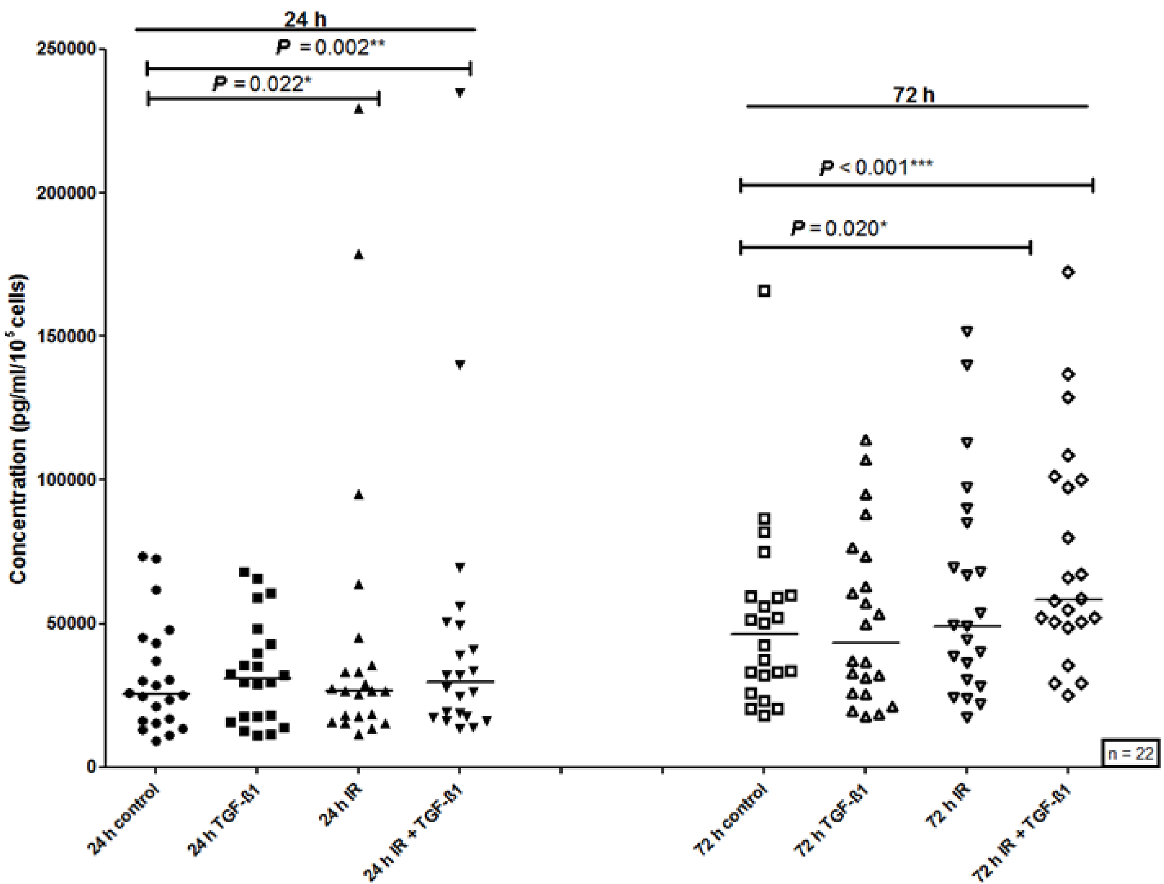
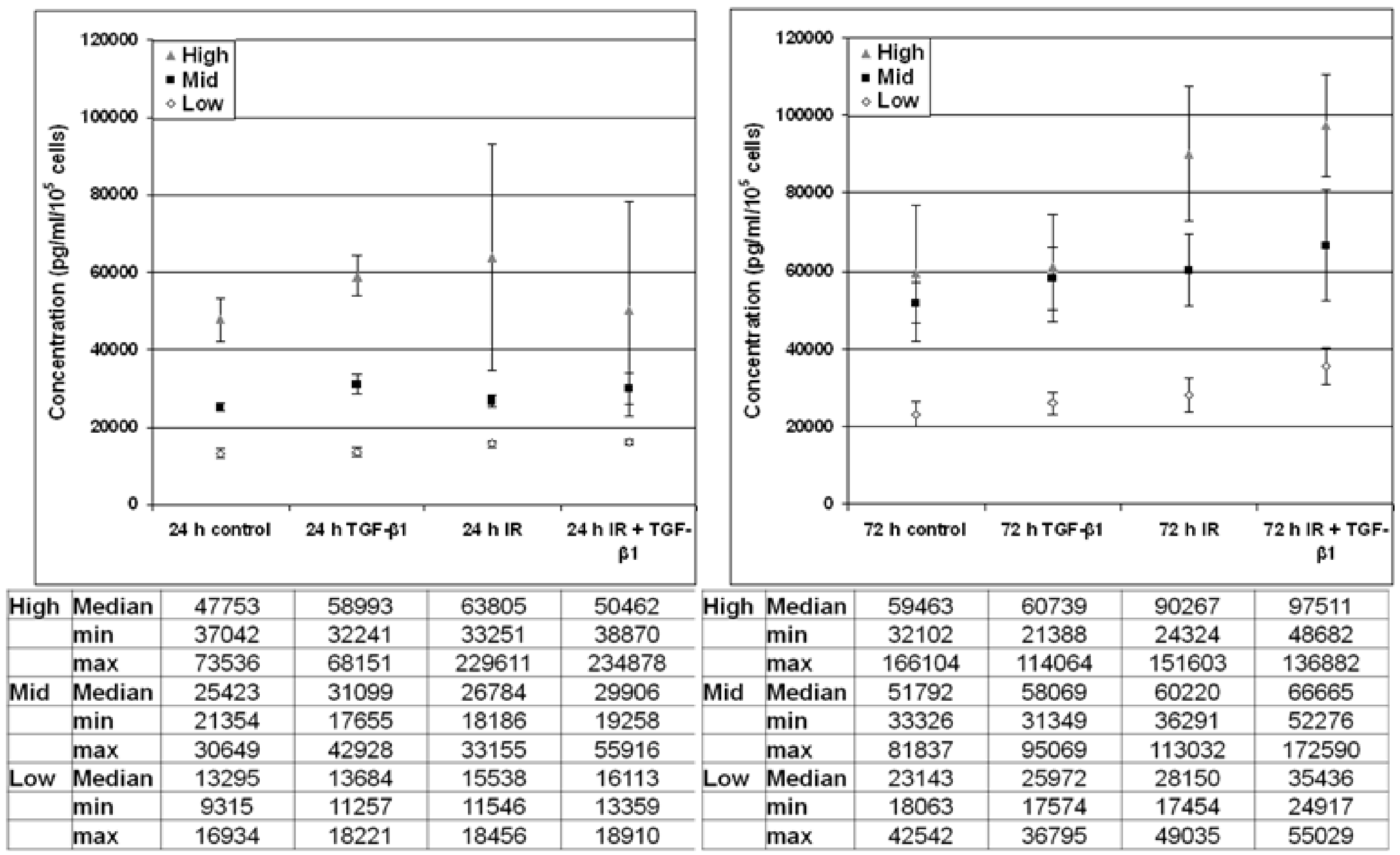


Figure 12. Primary fibroblast TIMP-1 secretion increases with IR treatment alone and TGF-β1 + IR treatment (n = 22). Primary human skin fibroblasts starved for 24 h were treated or not (control) with human TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 followed by ionizing radiation, then cultured for 24 h or 72 h. TIMP-1 secretion was determined by ELISA in culture supernatant. Bars denote median TIMP-1 secretion. TIMP-1=tissue inhibitor of metalloproteinase-1, TGF-β1=transforming growth factor-β1, IR=ionizing radiation, P calculated with Signed Ranks test using difference compared to control.



		24 h control	24 h TGF-β1	24 h IR	24 h IR + TGF-β1
High	Median	47753	58993	63805	50462
	min	37042	32241	33251	38870
	max	73536	68151	229611	234878
Mid	Median	25423	31099	26784	29906
	min	21354	17655	18186	19258
	max	30649	42928	33155	55916
Low	Median	13295	13684	15538	16113
	min	9315	11257	11546	13359
	max	16934	18221	18456	18910

		72 h control	72 h TGF-β1	72 h IR	72 h IR + TGF-β1
High	Median	59463	60739	90267	97511
	min	32102	21388	24324	48682
	max	166104	114064	151603	136882
Mid	Median	51792	58069	60220	66665
	min	33326	31349	36291	52276
	max	81837	95069	113032	172590
Low	Median	23143	25972	28150	35436
	min	18063	17574	17454	24917
	max	42542	36795	49035	55029

Figure 13. Behavior of low, mid, and high baseline TIMP-1 secreting primary fibroblast populations. Using baseline TIMP-1 secretion levels at 24 h under control condition as determined by ELISA, 22 patient-derived fibroblast samples were separated into 3 groups: low (n = 7), mid (n = 8), and high (n = 7). Median TIMP-1 secretion response of each group to treatments at 24 h and 72 h after treatment are shown as above. Values shown in pg/mL/10^5 cells. Error bars based on standard error. TGF-β1=transforming growth factor-β1, TIMP-1=tissue inhibitor of metalloproteinase-1, IR=ionizing radiation.

3.6 Active cathepsin K expression in primary skin fibroblasts

Active cathepsin K is a potent intracellular modulator of collagen type I degradation, which occurs following internalization of extracellular collagen type I by fibroblasts. Cathepsin K was detected by Western blotting in both its inactive form (zymogen) and the active form. As shown in Figure 14, inactive cathepsin K was detected at approximately 37 kDa, while the cleaved active form of cathepsin K was detected at approximately 25 kDa. Figure 15 shows the baseline expression of active cathepsin K at 24 h and 72 h of culture as well as baseline collagen type I secretion. Of 22 patient-derived fibroblast samples analyzed for cathepsin K expression, 11 expressed active cathepsin K. Interestingly, 8 out of the 11 patient-derived fibroblast cultures expressing the active form (72.7%) also had collagen type I secretion below the median value of collagen type I production. Furthermore, 9 out of 11 patient-derived fibroblast cultures with collagen type I levels above the median value did not express baseline active cathepsin K (81.8%) at either 24 h or 72 h in culture (Fig. 15). This suggests a trend of active cathepsin K expression accompanying low collagen type I production, perhaps an indication of the collagenolytic nature of cathepsin K in collagen type I regulation.

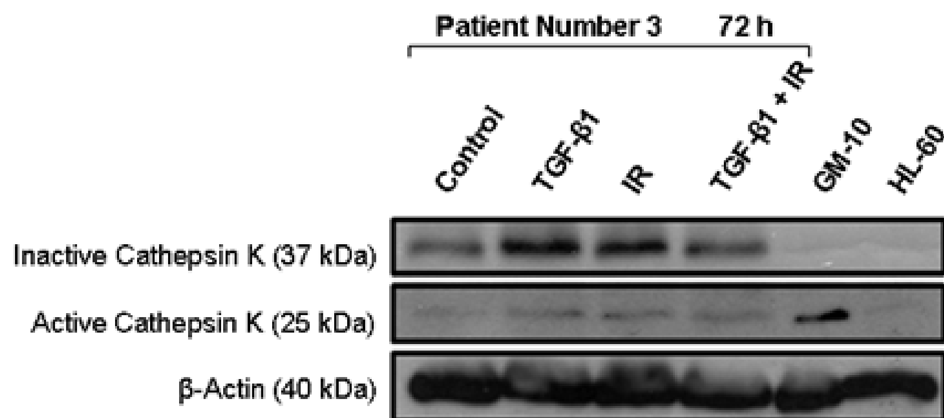


Figure 14. Active and inactive cathepsin K by Western blotting. Representative immunoblot of primary skin fibroblast lysate (patient number 3) collected 72 h without (control) and after treatment with TGF-β1 (1 ng/mL), ionizing radiation (2 Gy), or TGF-β1 combined to ionizing radiation. Lysates were subjected to immunoblot analysis using an antibody to detect both inactive and active cathepsin K forms at the molecular weights shown above. Actin was used to normalize for equal loading. GM-10 human fetal fibroblast lysate was used as a positive control, while HL-60 human leukemia cell lysate was used as a negative control for active cathepsin K. TGF-β1=transforming growth factor-β1, IR=ionizing radiation.

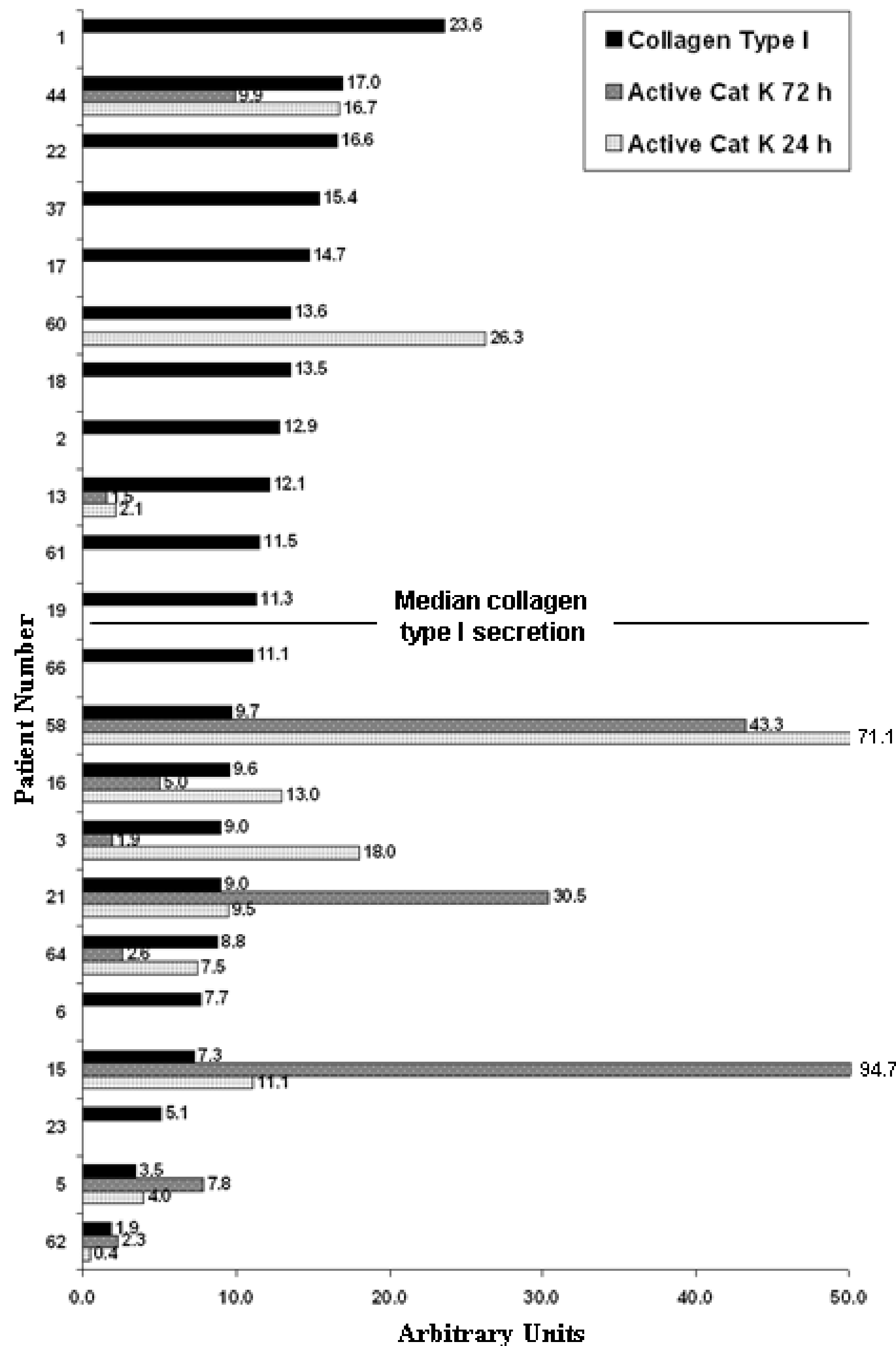


Figure 15. Baseline active cathepsin K expression and collagen type I levels of 22 primary skin fibroblast samples. Collagen type I levels determined by ELISA in fibroblast supernatant 72 h after treatment shown in arbitrary units. Cathepsin K expression values obtained by densitometry on bands detected by Western blotting of fibroblast lysate collected 24 h and 72 h after treatment. Cathepsin K shown in arbitrary units. Cat K=cathepsin K.

3.7 TGF-β1 treatment alone and TGF-β1 and IR combined treatment induced changes in collagen type I regulation and production

Table 6 shows that of the treatment conditions that patient-derived fibroblast cultures have been subjected to, TGF-β1 and the combined treatment of TGF-β1 and IR elicited the most substantial changes in collagen type I levels and secretion of factors involved in its regulation, i.e, TGF-β1, VEGF, and collagenolytic TIMP-1, and MMP-1. Treatment with TGF-β1 elicited an increase in median collagen type I production at 72 h post-treatment that was accompanied by an early and concurrent increase in VEGF secretion at both 24 h and 72 h and a decrease in MMP-1 secretion at 72 h, as well as an early induction of TGF-β1 that was not maintained at 72 h and a small but significant 5% decrease in MMP-1 secretion at 24 h with a 34% decrease at 72 h. IR treatment did not affect TGF-β1 and VEGF secretion at 24 h and MMP-1 secretion at both time points, but induced only small increases TGF-β1 and VEGF secretion 72 h after treatment and small increases in TIMP-1 at both 24 h and 72 h without affecting collagen type I production at 72 h post-treatment. The increase in collagen type I production induced by the combined TGF-β1 and IR treatment was accompanied by increases in TGF-β1 secretion, VEGF secretion, and TIMP-1 secretion that persisted from the earlier time point of 24 h until 72 h post-treatment. Even though TGF-β1 treatment alone significantly decreased MMP-1 secretion at both 24 h and 72 h time points, the combined treatment failed to do so.

	TGF-β1 secretion (n = 22)	VEGF secretion (n = 22)	MMP-1 secretion (n = 22)	TIMP-1 secretion (n = 22)
	24 h	24 h	24 h	24 h
TGF-β1 (1 ng/mL)	21% increase (0.009**)	82% increase (<0.001***)	5% decrease (0.022*)	no sig. change
IR (2 Gy)	no sig. change	no sig. change	no sig. change	5% increase (0.022*)
TGF-β1 +IR	45% increase (<0.001***)	103% increase (<0.001***)	no sig. change	18% increase (0.002**)

	TGF-β1 secretion (n = 22)	VEGF secretion (n = 22)	MMP-1 secretion (n = 22)	TIMP-1 secretion (n = 22)	Collagen type I levels (n = 40)
	72 h	72 h	72 h	72 h	72 h
TGF-β1 (1 ng/mL)	no sig. change	96% increase (<0.001***)	34% decrease (<0.001***)	no sig. change	18% increase (0.006**)
IR (2 Gy)	15% increase (<0.001***)	14% increase (0.027*)	no sig. change	6% increase (0.020*)	no sig. change
TGF-β1 +IR	25% increase (<0.001***)	175% increase (<0.001***)	no sig. change	25% increase (<0.001***)	15% increase (0.014*)

Table 6. Median primary fibroblast production/secretion of collagen type I, TGF-β1, VEGF, MMP-1, and TIMP-1 after treatment. TGF-β1, VEGF, MMP-1, and VEGF secretion levels in fibroblast culture supernatant determined by ELISA 24 h and 72 h after each treatment. Collagen type I levels in fibroblast culture samples determined by ELISA 72 h after treatment. Differences compared to control shown with *P* based on Signed Rank test; TGF-β1= transforming growth factor-β1, IR=ionizing radiation, VEGF=vascular endothelial growth factor, MMP-1=matrix metalloproteinase-1, TIMP-1=tissue inhibitor of metalloproteinase-1.

3.8 Significant and positive correlation between TIMP-1 and collagen type I levels following treatment with TGF-β1 alone and combined TGF-β1 and IR

We raised the question whether collagen type I levels could be directly related to secretion of TGF-β1, VEGF, MMP-1, or TIMP-1 either at basal levels or following treatment with TGF-β1 and/or IR. To answer this question, we searched for statistically significant correlations between patient-derived fibroblast collagen type I levels at 72 h and the secretion of each factor at 24 h and 72 h for each treatment condition. Significant correlations at 24h may account for early effects of treatment on secreted factors potentially involved in the regulation of collagen type I levels. As shown in Table 7, among all factors tested, a barely significant positive correlation was detected between collagen type I levels at 72 h and TIMP-1 secretion 24 h following TGF-β1 treatment ($R = 0.424$, $P = 0.049$, $n = 22$). Another positive correlation was detected between collagen type I levels 72 h after the combined TGF-β1 and IR treatment and fibroblast TIMP-1 secretion 24 h after the same treatment ($R = 0.503$, $P = 0.017$, $n = 22$).

Collagen type I					
Treatment		TGF-β1 secretion	VEGF secretion	MMP-1 secretion	TIMP-1 secretion
Control 24 h	R	-0.136	0.112	-0.163	0.225
	P	0.548	0.620	0.468	0.315
Control 72 h	R	-0.016	0.149	-0.290	0.103
	P	0.944	0.508	0.190	0.647
TGF-β1 24 h	R	0.285	0.007	-0.106	0.424
	P	0.199	0.974	0.640	0.049
TGF-β1 72 h	R	0.263	0.037	-0.049	0.097
	P	0.238	0.869	0.830	0.669
IR 24 h	R	-0.243	0.378	-0.168	0.258
	P	0.276	0.083	0.455	0.247
IR 72 h	R	-0.211	0.344	-0.328	0.097
	P	0.345	0.118	0.137	0.669
TGF-β1 + IR 24 h	R	0.141	0.200	-0.010	0.503
	P	0.533	0.372	0.966	0.017
TGF-β1 + IR 72 h	R	0.141	0.224	-0.188	0.206
	P	0.531	0.317	0.402	0.359

Table 7. Correlation between TGF-β1, VEGF, MMP-1, and TIMP-1 secretion and collagen type I levels under all treatments (n = 22). Levels of each secreted factor detected in fibroblast supernatant by ELISA 24 h and 72 h after treatment were compared to collagen type I levels detected by ELISA in fibroblast culture supernatant 72 h without (control) and with each specified treatment. Comparison was performed using Spearman's rank correlation coefficients. R=Spearman's rank correlation coefficient, TGF-β1=transforming growth factor-β1, IR=ionizing radiation, VEGF=vascular endothelial growth factor, MMP-1=matrix metalloproteinase-1, TIMP-1=tissue inhibitor of metalloproteinase-1. *P* calculated using Spearman's rank correlation coefficients.

3.9 Correlation between increased incidence of erythema grade 2 in patients treated with RT and high levels of ex-vivo IR-induced collagen type I

CTC grading for radiation dermatitis consists of a number of categories including dry and moist desquamation, erythema, edema, bleeding, and pain [14]. Data for incidence of acute toxicity was collected prospectively and independently of experimental studies investigating patient-derived fibroblasts. We stated the hypothesis that incidence of acute toxicity is dependent upon fibroblast collagen type I levels. The relationship between primary fibroblast collagen type I levels under each treatment condition and the incidence of high CTC grade (grade 2-3) moist desquamation or erythema (grade 2) was investigated by separating the total population of primary fibroblasts (n=40) into high and low collagen type I-producing groups. After testing with groups separated based on median levels or different cut-off values, we subjectively determined that at separation into high and low collagen type I-producing groups with the cut-off value 1.42 units of collagen type I granted the most significant differences in incidence of acute toxicities. As summarized in Table 8, increased incidence of erythema grade 2 in patients was significantly dependent on patient-derived skin fibroblast collagen type I levels after IR treatment ($\chi 2$ value = 4.019, $P = 0.045$, n = 40).

Acute Toxicity		Treatment (72 h)			
		Control	TGF-β1	IR	TGF-β1+IR
CTC (grade 2-3)	χ^2	0.010	0.404	2.182	0.753
	P	0.919	0.525	0.140	0.385
Moist Desquamation	χ^2	0.657	0.476	0.127	0.395
	P	0.418	0.490	0.722	0.530
Erythema (grade 2)	χ^2	0.780	1.026	4.019	2.973
	P	0.377	0.311	0.045	0.085

Table 8. Collagen type I levels following ex-vivo treatment with ionizing radiation (2Gy) were significantly correlated to the occurrence of erythema (grade 2, n = 40). Incidence of acute skin toxicity events was prospectively assessed in early breast cancer patients treated with radiation therapy. Collagen type I levels detected by ELISA in patient-derived fibroblast culture supernatant 72 h after treatment were compared to the incidence of acute toxicity events. For each treatment, fibroblasts were separated into high and low collagen type I-expressing groups using an arbitrary threshold point. A null hypothesis stating that the incidence of acute toxicity events does not differ between both groups was tested using the Pearson chi-squared test. $P < 0.05$ indicates that the null hypothesis is false. CTC=Common Toxicity Criteria, χ^2=chi squared test, TGF-β1= transforming growth factor-β1, IR=ionizing radiation.

Chapter 4. Discussion

Collagen type I and the growth factors and proteases involved in collagen type I regulation play central roles in wound healing and the pathological process of acute radiation-induced toxicity and long-term fibrosis. Some evidence of increased collagen type I as a consequence of ionizing radiation exposure has been provided in earlier studies using porcine skin models and human skin suction blister models [17, 42]. As significant producers of collagen type I in healthy skin and radiation-induced skin wounds, fibroblasts and the activated myofibroblasts have been examined to identify changes in the balance of collagen type I synthesis and regulation induced by ionizing radiation and growth factor simulation [46]. Investigating collagen type I regulation is of great significance, though it is challenging to analyze the aspects of this regulation in the context of RT-induced acute toxicity in breast cancer patients. Previous investigations focused mainly on collagen type I expression in patient skin months to years after RT [42, 43]. In a previous study, Illsley *et al.* had investigated the effect of irradiation on human skin fibroblast collagen production using fibroblasts derived from 5 patients treated *in vivo* with a single 8 Gy fraction [46]. This approach cannot be used in studies with a large sample since fibroblasts subjected to radiation of such dosage will be prone to senescence. In our study, we examined collagen type I expression and secretion in primary human skin fibroblasts derived from 40 early stage breast cancer patients using treatments mimicking the conditions of exposure to clinically relevant doses of IR with and without simultaneous treatment with TGF-β1, and regulation of collagen type I through a

number of factors expressed and/or secreted by skin fibroblasts: TGF-β1, VEGF, MMP-1, TIMP-1, and cathepsin K. We derived skin fibroblasts from 40 patients before initiation of their RT treatment schedule and performed quantitative and statistical analysis to understand the effect of IR and/or TGF-β1 with respect to changes in collagen type I production and its regulation ex-vivo.

4.1 Primary human skin fibroblast collagen type I production is more effectively induced by TGF-β1 treatment than IR

TGF-β1 is recognized as the major growth factor responsible for the radiation-induced fibrotic response in normal tissue [90] and is able to initiate intracellular signaling pathways to induce collagen type I synthesis, secretion, and deposition into the ECM by human skin fibroblasts [60]. We show that TGF-β1 treatment and the combined treatment of TGF-β1 and IR increased collagen type I levels in patient-derived fibroblasts (Table 6). The same treatments also induced changes in TGF-β1, VEGF, MMP-1, and TIMP-1 secretion. The autocrine effect of TGF-β1 treatment observed at 24 h after treatment is consistent with the positive feedback activity of TGF-β1 described in the literature [91]. Increased collagen type I levels following stimulation of patient-derived skin fibroblasts by TGF-β1 is consistent with data from a previous study reporting increased collagen type I synthesis by *in vitro* treatment of human fibroblasts derived from the skin of 5 patients with different types of cancer [46]. The most effective stimulus for primary skin fibroblast collagen type I regulation, at least in early response (up to 72 h in our study), was exogenous TGF-β1 rather than ionizing radiation. Previous studies reporting increased collagen type I synthesis after irradiation has

assessed collagen type I production through detection of collagen type I propeptides in suction blister fluid [42, 43, 92] or skin-derived fibroblasts isolated from patient skin [46] irradiated months to years prior to the time of study. In the present study, collagen type I production was assessed in fibroblast cultures 72 h after treatment with a clinically relevant dose of irradiation of 2 Gy with the purpose of detecting early changes in collagen type I levels that could be significantly associated with the onset of acute skin toxicity events. This may potentially identify an early predictive biomarker of RT-induced acute skin toxicity. The inference that TGF-β1 treatment was the primary and most effective source of stimulation of fibroblast collagen type I production can be applied to the observation that the combined treatment of TGF-β1 and a dose of 2 Gy irradiation elicited a median response similar in magnitude to that seen after TGF-β1 treatment alone. Hence, the bulk of the fibroblast collagen type I response to combined treatment was due to TGF-β1 stimulation, while IR stimulation was responsible for little, if any, increase in collagen type I production.

TGF-β1 induces collagen type I synthesis through the direct binding of active TGF-β1 to receptors on the fibroblast cell surface, which then transmits an intracellular signaling event through Smad3 to induce transcription of collagen type I propeptides in human skin fibroblasts (Fig. 1) [60]. This may provide a molecular explanation for the effect of exogenous TGF-β1 stimulation. On the other hand, the primary fibroblast cellular environment during treatment with IR consists of serum-starved culture medium with minimal amounts of latent TGF-β1 possibly secreted by primary fibroblasts prior to irradiation. Treatment with

irradiation then activates latent TGF-β1 through generation of ROS, resulting in increased bio-available active TGF-β1. This was not sufficient to enhance collagen type I levels within the time point assessed in our study. Because our data provide evidence that IR significantly increases TGF-β1 levels 72 h following treatment, we speculate that the indirect effect of IR through increased TGF-β1 levels may potentially increase collagen type I levels at later time points not investigated in our study. The increase in TGF-β1 following exposure to IR with subsequent increase of collagen type I levels may have detrimental effects *in vivo*. Indeed, if this effect is not opposed by other factors involved in the negative regulation of collagen type I levels, the balance will be shifted towards an excessive accumulation of collagen type I in skin fibroblasts.

4.2 Increased VEGF secretion occurs in conjunction with increased collagen type I levels

The role of VEGF in the pathological process of radiation-induced toxicity and fibrosis involves the promotion of migration and tissue integration of different cell types as a new source of pro-inflammatory cytokines (such as TGF-β1) and accumulation of collagen type I into the radiation wound region [70]. A previous study had noted that application of a small molecule inhibitor of the VEGF receptor kinase reduced pro-fibrotic genetic markers such as procollagen I gene expression and *TGF-β1* gene expression in a murine pulmonary fibrotic model [93]. It is therefore interesting to note that, in our study, increased fibroblast collagen type I production is accompanied by significantly increased VEGF secretion at both early (24 h) and late (72 h) time points. In fact, the only

treatment condition that did not induce collagen type I production (IR treatment) failed to induce early VEGF secretion and had moderate effects on VEGF secretion at 72 h (14% under IR treatment compared to 96% increase under TGF-β1 treatment, and 175% increase under combined treatment, Table 6). The clinical relevance of these findings reside in the fact that patients treated with IR who also secrete higher levels of TGF-β1 may have further deleterious effects through increased secretion of VEGF, promoting the maintenance and expansion of the collagen type I-producing population within a radiation wound region.

Our correlative studies indicate a lack of statistical correlation between collagen type I production and VEGF secretion. This could be due to the high inter-individual variability between patients with respect to VEGF secretion and/or to the fact that our *in vitro* experimental model may not account for an indirect relationship between VEGF and collagen type I. The molecular mechanism for the induction of fibroblast VEGF secretion as indicated in the literature (Fig. 2) offers explanation for our experimental observations. Our data indicates increased median fibroblast VEGF secretion by TGF-β1 treatment by approximately 2-fold at both 24 h and 72 h time points (Table 3) are similar to the data obtained by a previous study using the same TGF-β1 treatment conditions, which resulted in a near 3-fold increase in VEGF secretion by human fetal lung fibroblasts at 24 h post-treatment [73]. It is interesting to note that increased primary fibroblast VEGF secretion was most often accompanied by increased TGF-β1 secretion (Table 6). Further correlative statistical analysis to identify a possible correlation between VEGF levels and TGF-β1 levels is warranted.

4.3 Disruption of MMP-1/TIMP-1 balance accompanies increased primary fibroblast collagen type I levels

Degradation of extracellular collagen type I is mediated by MMP-1, or interstitial collagenase [94], which cleaves collagen type I and is inhibited by direct binding of the active site by TIMP-1 [81]. This interaction of the protease and its inhibitor suggests that a balance exists between MMP-1 and TIMP-1 when synthesis and deposition of extracellular collagen type I within the ECM is maintained at equilibrium. A pathogenic state of radiation-induced injury and fibrosis would shift this balance towards reduced MMP-1 activity and subsequent increased collagen type I accumulation. In our study, this imbalance seems to characterize the response of primary fibroblasts to the TGF-β1 treatment and the combined TGF-β1 and IR treatment which significantly increased collagen type I levels. A previous study using human neonatal skin fibroblasts determined that MMP-1 protein expression was decreased by approximately 70% 24 h after treatment with a relatively high dose of TGF-β1 (12.5 ng/mL) [80]. In our study, we used a lower dose of TGF-β1 (1 ng/mL) but still observed a decrease in primary human skin fibroblast MMP-1 secretion. This observation may provide a molecular explanation for the concurrent increase in collagen type I production 72 h after treatment, as the MMP-1/TIMP-1 balance has shifted towards decreased collagenolytic activity. Extremely high doses of irradiation (30-64 Gy) have been reported to decrease MMP-1 expression while increasing TIMP-1 expression [17]. We did not observe a similar trend with our relatively low dose of 2 Gy. Although IR treatment elicited small but significant increases in TIMP-1 secretion,

it did not alter MMP-1 collagenolytic activity and did not significantly affect collagen type I levels at the time point analyzed in our study. A larger and more significant increase in TIMP-1 secretion induced by combined TGF-β1 and IR treatment accompanied increased primary fibroblast collagen type I levels, once again suggesting that the MMP-1/TIMP-1 balance supporting the collagen type I equilibrium has been disrupted. Correlation analysis revealed that while MMP-1 secretion was not directly correlated to collagen type I production, early increases in TIMP-1 secretion in response to TGF-β1 treatment and combined TGF-β1 and IR treatment were correlated to increases in collagen type I secretion. This suggests that primary fibroblast production and accumulation of collagen type I in response to TGF-β1 and/or IR treatment is directly related to increased TIMP-1 levels. Hence the role of TIMP-1 as an inhibitor of MMP-1 collagenolytic activity has to be further investigated in clinical studies to assess the relevance of our findings in the context of RT-induced acute toxicities.

4.4 Expression of active cathepsin K possibly related to low collagen type I levels?

Cathepsin K in an intracellular protease that degrades collagen type I prior to release of collagen type I from the cell and following uptake of extracellular collagen type I from the ECM by fibroblasts [88]. Our observations of patient-derived skin fibroblast cathepsin K expression indicate that certain patient fibroblasts tend to more readily express active cathepsin K. The majority of those patient-derived fibroblast cultures that expressed active cathepsin K were among the population with collagen type I production levels below median values, while

most of the patient-derived fibroblast cultures that did not express active cathepsin K were above median collagen type I levels. This suggests that cathepsin K prevents collagen type I accumulation, which is critical for radiation-induced toxicities and fibrosis [95]. In our study, we did not statistically determine the effect of our treatment conditions and correlations to collagen type I production, but we have identified a subset of patient-derived skin fibroblasts with more readily expressed active cathepsin K. Further expansion of the number of patient-derived skin fibroblast cultures assessed for active cathepsin K expression may prove of interest with respect to primary fibroblast collagen type I production and the relationship with acute toxicity outcome.

4.5 Contributions of skin fibroblast collagen type I regulation to radiation-induced toxicities

Our study allows us to postulate a model of skin fibroblast involvement in the development of radiation-induced toxicities. Due to time constraints, we were not able to answer the question of whether our secreted factors are correlated to each other. Once this analysis has been performed, we will be able to identify possible correlations between increased primary fibroblast TGF-β1 secretion and secretion of VEGF, MMP-1, and TIMP-1. This would possibly illustrate the role of TGF-β1 as the key mediator in our proposed model of collagen type I regulation by emphasizing the potent ability of TGF-β1 to modulate secretion of other collagen-regulatory factors. Our data in the context of a radiation wound resulting from RT support our proposed model of collagen type I regulation as illustrated in Figure 3. The repeated insults of fractionated radiation doses would

result in induction of increased latent TGF-β1 levels in skin fibroblasts. Consecutive doses of IR would then continuously activate a constantly replenishing supply of fibroblast-secreted latent TGF-β1 maintained and prolonged by radiation-activated TGF-β1 stimulation. Fibroblasts would contribute to a TGF-β1-stimulated increase in VEGF concentration within the wound site, leading to vasculature permeability and migration of blood-borne fibroblast precursors to the wound region. As suggested by our observations on primary skin fibroblast MMP-1 and TIMP-1 secretion, a wound environment enriched in TGF-β1 and under continuous radiation stress would shift the MMP-1/TIMP-1 balance towards a decrease in extracellular collagen type I degradation and accumulation of collagen type I at the wound site. Another possible factor in modulation of collagen type I degradation is patients whose skin fibroblasts less readily express active cathepsin K, which may further lead to collagen type I deposition and accumulation. Each of these factors may contribute to promoting and maintaining increased fibroblast collagen type I production and deposition, thus increasing the possibility of developing radiation-induced toxicities and fibrosis.

4.6 Collagen type I response to ionizing radiation is related to incidence of erythema in early stage breast cancer patients

Using patient acute toxicity data collected through an independent prospective clinical study, we statistically investigated relationships between patient-derived skin fibroblast collagen type I production and indicators of acute radiation-induced toxicity including CTC grade, moist desquamation, and

erythema. Although CTC grade is considered to be a comprehensive indicator of radiation-induced toxicity encompassing other indicators including moist desquamation and erythema, we did not find a relationship between fibroblast collagen type I production and either CTC grade or moist desquamation. However, we did find that IR treatment-induced increase in primary fibroblast collagen type I production is significantly related to incidence of grade 2 erythema. Because of the rate of acute skin toxicities (~40% of the population analyzed) and because of possible subjective rating of erythema, we need to expand our study population to further investigate this possible relationship between acute toxicity, collagen type I levels and/or other effectors of collagen type I regulation.

4.7 Perspective: Sources of collagen type I producing fibroblast/ myofibroblast population in radiation-induced fibrosis

The initiation and progression of radiation-induced toxicities involves highly active sub-populations of collagen type I-secreting fibroblasts and myofibroblasts. The mechanism of activation of these proposed sources involve fibroblast secreted factors responsible for collagen type I regulation, indicating the potential for positive feedback processes due to an increase of collagen type I-secreting cells, further emphasizing the role of these factors as key mediators in collagen type I regulation and radiation-induced toxicity.

4.7.1 Resident fibroblast activation

Activation of local tissue fibroblasts within the region of injury were originally believed to be the primary source of myofibroblasts in wound healing [96]. In radiation-induced injury, resident fibroblasts are likely to be among the first cellular factors influenced by the release of stored cytokines from the ECM including TGF-β1 [97], activating Smad3 signaling [98] and the mitogen-activated protein kinase (MAPK) signaling pathway [99] to induce the expression of α-SMA in fibroblasts, a marker for myofibroblasts [100]. In radiation-induced toxicity, fibroblast activation is likely supported and maintained by exogenous TGF-β1 production by cells within the wound region as well as autocrine fibroblast stimulation [15]. This involvement of TGF-β1 stimulation in fibroblast activation may provide new insights in our study, keeping in mind that we found collagen type I levels to be increased after TGF-β1 treatments. It is possible that the TGF-β1 treatment initiated fibroblast activation and is triggering the transition of our primary fibroblasts into myofibroblasts. It would be of great interest to investigate α-SMA expression in our patient-derived skin fibroblasts after TGF-β1 treatment as an indicator of fibroblast activation.

4.7.2 Epithelial to mesenchymal transition

Epithelial cells may also be an important source of myofibroblasts through EMT, a possible event during wound healing [101]. EMT is the process through which epithelial cells phenotypically undergo a transition into mesenchymal cells [102], acquiring mesenchymal markers such as α-SMA. A murine renal model found 36% of fibrotic disease-related fibroblasts stemmed from tubular epithelial

origin [103], suggesting that EMT is a strong proponent of radiation-induced fibrosis. Among the more potent EMT induction signals is TGF-β1 through Smad3 signaling [50, 104, 105]. This activity of TGF-β1 suggests a role for fibroblasts in promoting EMT during the process of radiation-induced toxicity, since primary skin fibroblast TGF-β1 secretion was increased following IR and TGF-β1 combined treatment. Also, from our observation that a high TGF-β1-secreting population exists among our patient-derived fibroblasts (Fig. 7), we may postulate that TGF-β1-induced EMT may have a more detrimental effect *in vivo* for this sub-group of patients.

4.7.3 Circulating bone marrow-derived cells

A systemic source of fibroblasts and myofibroblasts may exist in circulation as bone marrow-derived fibroblast precursors [106] including what has been termed 'fibrocytes,' first identified as unique circulating cell types that specifically enter sites of injury and express collagen type I. These fibrocytes are adherent, spindle-shaped cells resembling fibroblasts but unlike fibroblasts, expressed the leukocyte marker CD34 [107]. Since their discovery, fibrocytes have been shown to be recruited to bleomycin-induced fibrotic lung injuries [108], and to be induced by TGF-β1 to express α-SMA and gain myofibroblast-like contractile ability in murine and human *in vitro* models [109]. Because of increased primary fibroblast TGF-β1 secretion following TGF-β1 and IR treatment, fibroblasts may play a role in the transformation of fibrocytes into collagen type I-producing myofibroblast-like cell types. Exploration of α-SMA

expression in isolated circulating fibrocytes from peripheral blood and cultured in fibroblast-conditioned medium (source of TGF-β1) may further elucidate this role.

4.8 Conclusion and future directions

Previous studies investigating the effect of irradiation on collagen type I levels in the skin of cancer patients often involved analysis of procollagen type I concentrations in suction blister fluid or culture of fibroblasts derived from tissue irradiated months to years previously. Their studies were often limited by the small sample size of the patient population. Here, we demonstrate the feasibility of our approach using primary fibroblasts derived from non-irradiated skin obtained from patients before the start of RT and investigating the effect of *ex vivo* IR on fibroblast collagen type I regulation with exposure to IR at a clinically relevant dose. In its current form, our study is limited by sample size, especially in relation to acute radiation-toxicity since only a fraction of patients develop toxicity events. With additional patient-derived fibroblast samples archived and patient outcome data collected in a prospective manner, our sample size can be further expanded. Also, the scope of this investigation could be expanded through inclusion of other pro-inflammatory cytokines up-regulated in a wound healing response such as TNF-α, IL-1, and IL-6 [9] using the same patient-derived fibroblast samples.

The statistical analysis described in this study is still in a preliminary stage. Correlation between secreted factors and collagen type I have been investigated, but correlations between individual secreted factors are yet to be considered. Of key interest is the relation between fibroblast TGF-β1 secretion and VEGF,

MMP-1, and TIMP-1 secretion, which may give further indication of TGF-β1 modulation of those factors in the progression of radiation injury. The relationship between patient-derived fibroblast collagen type I production and regulation may also be further explored. With expansion of our patient sample size, it may be possible to correlate multiple factors of fibroblast behaviour with incidence of acute and late radiation-toxicity events in patients. To establish a model of collagen type I regulation based on our experimental findings, our statistical analysis investigating the relationship between fibroblast collagen type I production and secreted factors may also be extended to analyzing the relationship between collagen type I levels and a combination between different secreted factors.

Our findings indicate that primary human skin fibroblast collagen type I production and its regulation is modulated by IR and exogenous TGF-β1 stimulation, suggesting that fibroblasts play a critical role in the collagen type I overproduction involved in radiation-induced toxicity and fibrosis. We observed that the response of fibroblasts to treatment was dominated by TGF-β1 stimulation, while IR elicited relatively less fibroblast response in factor secretion and collagen type I production. This may be an indication of a critical role for TGF-β1 in determining the resolution of a radiation-induced injury after RT. To better understand the full scale of this fibroblast collagen type I response, further studies involving larger patient sample sizes and additional correlative statistical analyses are required, which may elucidate predictive markers for indicators of acute and late radiation-induced toxicity such as CTC grade and fibrosis.

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