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Review

TGF-β in radiotherapy: Mechanisms of tumor resistance and normal tissues injury

Bagher Farhood[a], Ehsan khodamoradi[b], Mojtaba Hoseini-Ghahfarokhi[b], Elahe Motevaseli[c], Hanifeh Mirtavoos-Mahyari[d], Ahmed Eleojo Musa[e], Masoud Najafi[b,*]

[a] Department of Medical Physics and Radiology, Faculty of Paramedical Sciences, Kashan University of Medical Sciences, Kashan, Iran
[b] Radiology and Nuclear Medicine Department, School of Paramedical Sciences, Kermanshah University of Medical Sciences, Kermanshah, Iran
[c] Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[d] Lung Transplantation Research Center (LTRC), National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
[e] Department of Medical Physics, Tehran University of Medical Sciences (International Campus), Tehran, Iran

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ABSTRACT

Emerging evidences show that changes in tumor stroma can adapt cancer cells to radiotherapy, thereby leading to a reduction in tumor response to treatment. On the other hand, radiotherapy is associated with severe reactions in normal tissues which limit the amount radiation dose received by tumor. These challenges open a window in radiobiology and radiation oncology to explore mechanisms for improving tumor response and also alleviate side effects of radiotherapy. Transforming growth factor beta (TGF-β) is a well-known and multitasking cytokine that regulates a wide range of reactions and interactions within tumor and normal tissues. Within tumor microenvironment (TME), TGF-β is the most potent suppressor of immune system activity against cancer cells. This effect is mediated through stimulation of CD4+ which differentiates to T regulatory cells (Tregs), infiltration of fibroblasts and differentiation into cancer associated fibroblasts (CAFs), and also polarization of macrophages to M2 cells. These changes lead to suppression of cytotoxic CD8 + T lymphocytes (CTLs) and natural killer (NK) cells to kill cancer cells. TGF-β also plays a key role in the angiogenesis, invasion and DNA damage responses (DDR) in cancer cells. In normal tissues, TGF-β triggers the expression of a wide range of pro-oxidant and pro-fibrosis genes, leading to fibrosis, genomic instability and some other side effects. These properties of TGF-β make it a potential target to preserve normal tissues and sensitize tumor via its inhibition. In the current review, we aim to explain the mechanisms of upregulation of TGF-β and its consequences in both tumor and normal tissues.

1. Introduction

Tumor resistance is one of the main challenges of cancer therapy. Resistance of tumor to radiotherapy increases the need for higher radiation doses, which is associated with normal tissue toxicity [1]. In fact, normal tissue injury is the main limiting factor for the delivery of sufficient dose of ionizing radiation to tumor [2]. Although tumor radiotherapy is associated with normal tissue injury, this should be acceptable without causing severe damages to normal functions of organs. This issue is even more critical for highly radiosensitive organs. For example, radiotherapy of pelvis or chest cancers can affect the activity of bone marrow stem cells, leading to a reduction of peripheral blood cells, especially lymphocytes and platelets. Severe damages to the bone marrow can attenuate immune system functions which lead to infection and bleeding. This can cause severe side effects and may halt the course of treatment [3]. Some other important side effects include acute inflammation in the lung, heart, brain and gastrointestinal system, which affect patients' quality of life years after the end of radiotherapy or even lead to death [4].

Minimizing normal tissue injury with efficient delivery of ionizing radiation to tumor is a critical issue in radiotherapy [5]. Although some technological advancements in recent decades have helped improve localized radiotherapy, however, side effects to normal tissues and tumor recurrence after radiotherapy remain critical problems [6]. The knowledge of how normal tissues react to ionizing radiation can improve the management of early and late side effects of radiotherapy. Furthermore, the knowledge of tumor architecture as well as the cellular and molecular mechanisms of tumor radioresistance can help us to

* Corresponding author.
E-mail address: najafi_ma@yahoo.com (M. Najafi).

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suggest new approaches to increase response to radiotherapy and reduce probability of tumor recurrence [7].

Evidences show that ionizing radiation can change the normal level of several factors such as cytokines in the body, which play a key role in the progression of radiation-induced normal tissue injury [8]. Similar effects have also been observed in tumor [9]. In fact, although ionizing radiation kills cancer cells, responses from tumor microenvironment (TME) to radiation and cell death adapt cancer and cancer stem cells to subsequent doses of radiotherapy [7]. This causes reduction in the efficiency of radiotherapy while it amplifies normal tissue toxicity. One of the most important factors that act in this way is transforming growth factor (TGF)-β [10]. In the current paper, we aim to review the mechanisms of upregulation of TGF-β following exposure of cells to radiotherapy. Furthermore, we explain cellular and molecular effects of TGF-β in both tumor resistance to radiation and damage to normal tissues.

1.1. TGF-β signaling

TGF-β includes three isoforms: TGF-β1, TGF-β2, TGF-β3, with three types of receptors, TβR1, TβR2, and TβR3 [11]. It seems that TGF-β1 plays a central role in normal tissue injury and tumor resistance following radiotherapy. TGF-β inserts its effect through two known pathways including canonical (Smad-dependent) and non-canonical (Smad-independent) pathways [12].

1.2. Canonical pathway

After extracellular upregulation of TGF-β, it can bind to TβR2 or TβR3. TβR3 can form a heteromeric complex with TβR2. TβR2 finally interacts with TβR1 which is associated with activation of TβR1. The activated TGF-β receptors trigger phosphorylation of Smad2 and Smad3 heterodimers. Smad2 and Smad3 in association with Smad4 develop a complex that translocates into the nucleus and can regulate several genes. Canonical pathway plays a central role in the regulation of fibrosis genes such as collagen I, collagen III, fibronectin, and α-SMA [13].

1.3. Non-canonical pathway

Smad2/3 dependent pathways have potent interactions with other signaling pathways that play key roles in the consequences of exposure to external stimuli and activation of TGF-β signaling. However, TGF-β interaction with its receptors can trigger regulation of non-Smad pathways such as Rho/ROCK, p38, JNK *etc*. [14]. Stimulation of Rho/ROCK pathway by TGF-β plays a key role in regulating the fibrosis process [15]. Although TGF-β is known as an anti-inflammatory growth factor, the interaction between TβR1 and TβR2 can induce upregulation of TRAF4 and TRAF6, which trigger inflammatory mediators such as NFκB, a multifunctional transcription factor [14]. NFκB can induce the expression of COX-2, iNOS, STAT3 *etc*., which are involved in inflammation, fibrosis and EMT [16]. This pathway also has a potent effect on apoptosis resistance of cancer cells [16]. TβR1/TβR2 also can induce Ras and MAP3K7, result in upregulation of MAPKs, including JNK p38, ERK. This pathway also can induce redox interactions, proliferation and epithelial-to-mesenchymal transition (EMT), which cause both damages to normal tissues and tumor resistance to therapy [15]. (Fig. 1)

1.4. Ionizing radiation triggers release of TGF-β

TGF-β is a multitasking cytokine that regulates proliferation, cell death and management of immune system responses [17]. It is one of the most common cytokines that is released following exposure of cells to ionizing radiation. In contrast to some other cytokines, the release of TGF-β has a direct relation with radiation dose [18]. This may indicate that TGF-β is a critical marker of radiation toxicity and DNA damage in irradiated cells. DNA damage, which is the first genetic consequence of ionizing radiation in cells, leads to activation and upregulation of TGF-βII [19]. It has been suggested that upregulation of both TGF-βI and TGF-βII are involved in DNA damage responses (DDRs) in cells following exposure to clastogenic agents [20]. Furthermore, radiation-induced cell death plays a key role in the release of TGF-β and subsequent consequences. Radiation can kill cells through different mechanisms such as apoptosis, mitotic catastrophe, necrosis, senescence and autophagy. Clearance of dying cells which undergo each of these death mechanisms has different consequences. For example, apoptotic bodies' clearance is done by macrophages, while necrotic cells release damage-associated molecular patterns (DAMPs) to trigger lymphocytes. Clearance of apoptosis by macrophages leads to the release of anti-inflammatory cytokines including TGF-β and IL-10. However, DAMPs can trigger toll like receptors (TLRs) to upregulate NFκB and release inflammatory cytokines [21].

Based on the mentioned mechanisms, the balance between apoptosis and necrosis has a key role in the release of TGF-β. Some organs and also some cancers such as bone marrow and its derived malignancies such as lymphoma, have high incidence of apoptosis. By contrast, some tumors like sarcomas may not show apoptosis after radiotherapy [22]. Another important factor that affects the incidence of apoptosis and necrosis is radiation dose. Using low doses of radiation can cause more apoptosis to necrosis ratio. This is obvious for doses of radiation lower than 1 Gy. Hyperfractionated radiotherapy, which involves some fractions per day, uses low radiation doses. However, conventional and hypofractionated radiotherapy have higher doses per fraction. The different consequences from each of these techniques may be related to various types of cell death [23].

In addition to apoptosis, senescence is another major inducer of TGF-β. Senescence of both normal and cancer cells have been shown to trigger release of TGF-β [24,25]. Induction of DNA damage by ionizing radiation leads to upregulation of DNA repair enzymes such as p53, which may mediate senescence [26]. Furthermore, continuous ROS generation following mitochondrial malfunction or overexpression of NADPH oxidase enzymes plays a key role in the induction of senescence following exposure to ionizing radiation [27,28]. (Fig. 2)

In addition to monocytes and macrophages, platelets are another important source of TGF-β after radiotherapy. It has been confirmed that platelets exist in association with other inflammatory cells within fibrotic areas after exposure to ionizing radiation [29]. Platelets have a large amount of TGF-β1 in comparison to other immune cells, which play a central role in the development of radiation-induced fibrosis in organs such as the heart [30]. Knockdown of platelet derived TGF-β1 has been shown to reduce fibrosis significantly [29].

1.5. TGF-β in normal tissues injury

TGF-β is a regulator of wound healing. However, its overproduction after exposure to high doses of ionizing radiation can lead to severe injuries in normal tissues. It is well documented that an increase in TGF-β following exposure to ionizing radiation has a pivotal role in the progression of several side effects in different organs [31]. Fibrosis is the most common outcome following increase in TGF-β in normal tissues. However, nowadays, it is well known that TGF-β can be involved in some other mechanisms of normal tissue toxicity such as chronic oxidative stress and second primary cancers, myelosuppresion and heart failure [31]. In this section, we explain the mechanisms of TGF-β induced normal tissue injury following exposure to ionizing radiation.

1.6. TGF-β in radiation-induced fibrosis

Fibrosis is an unusual stiffness of tissues, which results from abnormal deposition of collagen in extracellular matrix [32]. TGF-β plays a pivotal role for progression of collagen and fibronectin deposition in

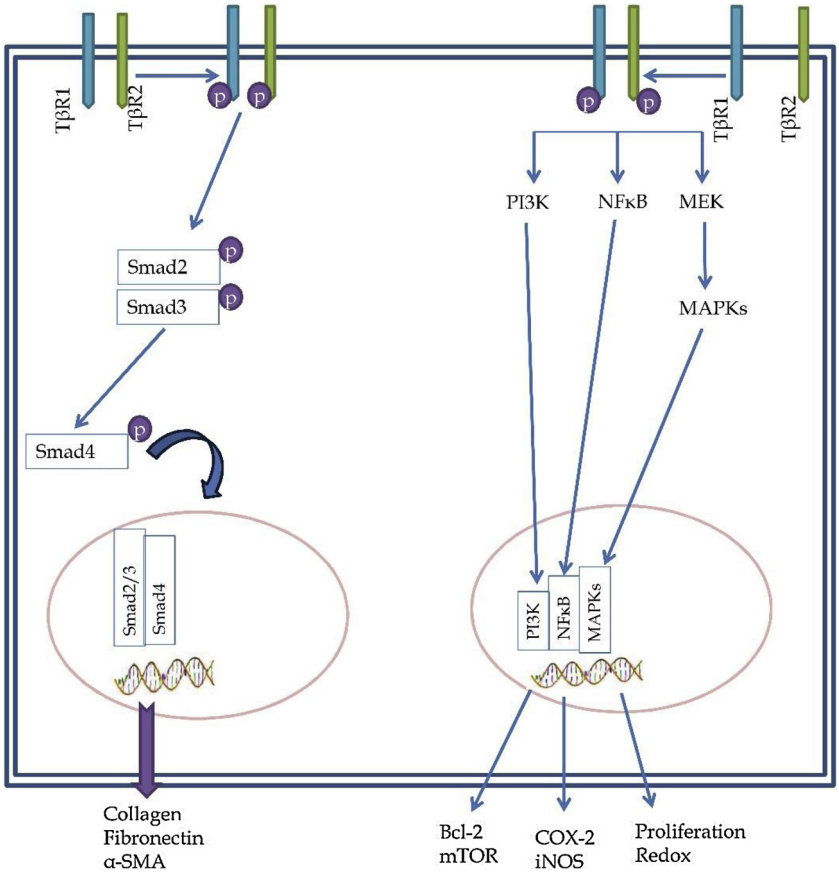

Fig. 1. TGF-β signaling including canonical and non-canonical pathways.

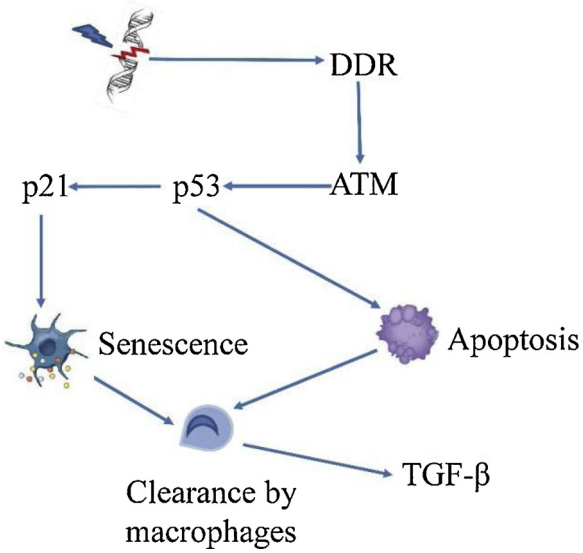

Fig. 2. Mechanisms of radiation-induced TGF-β activation. DNA damage can lead to cell death through different mechanisms. Among them, apoptosis and senescence are responsible to induce macrophages to release TGF-β.

extracellular matrix, leading to fibrosis. Studies have shown that TGF-β can promote fibrosis through various signaling pathways. The canonical pathway is mostly common known for promotion of fibrosis. However, non-canonical TGF-β signaling pathways including mir-21, Rho/ROCK and NADPH oxidase have a role in radiation-induced fibrosis [32].

1.7. Smad2/3 pathway

Smad2/3 is one of the most common signaling pathways that is involved in progression of radiation-induced fibrosis [33]. Important receptors of TGF-β including TβRI and TβRII enhance the expressions of Smad2 and Smad3. Knockdown of Smad3 has confirmed that TGF-β potentiates radiation fibrosis through this pathway [34]. Upregulation of Smad2/3 during the process of fibrosis, increase in the expressions of collagen I and III, vimentin and α-SMA following exposure to radiation, have been confirmed by several studies (Dai et al., 2019; [35,36]). It seems that upregulating some other mediators such as miR-21 and mTOR2 is involved in the induction of fibrosis through activation of Akt and Smad2/3 [37,38]. Targeting mTOR2 has shown that attenuate the expression of α-SMA and collagen in the lung. mTOR2 also help to progression of fibrosis via suppression of apoptosis in fibroblasts (Dai et al., 2019). MiR-21 is able to suppress Smad7, an inhibitor of Smad3 [39].

1.8. Rho/ROCK pathway

This pathway is involved in several functions including cytoskeleton and cell polarity [40]. This pathway plays a key role in wound healing [41]. The involvement of Rho/Rock pathway in the progression of fibrosis have been confirmed by some studies [42]. This pathway is known as a regulator of radiation-induced fibrosis following upregulation of TGF-β. Thus, targeting this pathway has been suggested for reducing side effects of radiotherapy. For the first time, it has been shown that Rho/Rock plays a key role in late fibrosis of the lung and heart tissues following irradiation [43]. Suppression of this pathway after irradiation has been shown to reduce the connective tissue growth factor (CTGF) and fibrogenesis process in intestinal cells [44]. This pathway also triggers the binding of NFκB to DNA [44]. Upregulation

and nuclear translocation of NFκB may further amplify fibrogenesis through stimulation of EMT and the expression of some pro-fibrosis genes such as COX-2 and other inflammatory mediators [45].

Inhibition of Rho pathway can attenuate the activities of fibroblasts [46]. Pravastatin is a drug that can inhibit Rho/Rock pathway. Using pravastatin has been shown to suppress EMT and upregulation of type I collagen and fibronectin following irradiation of rat's intestine. Pravastatin could suppress both inflammation and fibrosis in rat's intestine, thereby indicating the role of Rho/Rock pathway in the stimulation of both inflammatory and fibrotic processes [47]. A clinical trial confirmed that pravastatin ameliorates skin fibrosis in patients that underwent radiotherapy for head and neck cancers [48]. Selective inhibition of Rho shows that this pathway may be involved in the side effects of ionizing radiation through attenuation of DDR. A study showed that inhibition of Rho can enhance DNA repair capacity, thus reduces apoptosis. This was also associated with alleviation of lung fibrosis after irradiation [49].

1.9. NOXs pathway

Radiation-induced fibroblast senescence plays a key role in the activation of fibroblasts and fibrosis [50]. Senescence is a key regulator of fibrosis through activation of TGF-β–NOXs pathway. It has been observed that senescence caused by ionizing radiation-induced activation of NOX4 can also further activate TGF-β receptors I&II, leading to fibrosis through activation of canonical pathway [51]. ROS generation by NOX enzymes plays a key role in the upregulation of TGF-β and progression of radiation-induced fibrosis. Attenuation of NOX4 expression and also administration of antioxidants has been shown to reduce the expression of TGF-β and accumulation of extracellular matrix collagen [52].

1.10. MiR-21

MiR-21 is a critical regulator of several reactions in normal and cancer cells to ionizing radiation. In normal tissues, it can suppress antioxidant defense and also amplify fibrosis after radiotherapy. MiR-21 can be induced following upregulation of TGF-β [53]. However, overexpression of MiR-21 can increase the regulation of TGF-β following suppression of SOD2 and increase in oxidative stress. MiR-21 is able to potentiate TGF-β signaling, leading to enhancing fibrosis development [54]. Induction of MiR-21 after irradiation induces the upregulation of TGF-β–Smad2, while it reduces the expression of Smad7. Inhibition of MiR-21 could reverse this pathway and attenuate radiation-induced pulmonary fibrosis [54]. (Fig. 3)

1.11. TGF-β in chronic oxidative stress and genomic instability

Continuous production of ROS/NO is a common consequence of exposure to ionizing radiation in various cells/organs. Although ionizing radiation induces its detrimental effect mainly through production of ROS, changes in reduction/oxidation (redox) reactions after exposure to radiation play a key role in oxidative stress, genomic instability, carcinogenesis, inflammation and other side effects of ionizing radiation [55]. To date, several ROS sources have been known within cells. NADPH oxidases (NOXs) and dual oxidases (DUOXs), COX-2, inducible nitric oxide synthase (iNOS), lipoxygenases (LOX), endoplasmic reticulum, and mitochondria can generate free radicals during normal or pathological conditions [56]. Interestingly, most of these mediators are highly dependent on TGF-β level and the expression of its receptors [57]. These enzymes and organelles act in a positive feedback loop with each other, hence, increased ROS generation by one of these mediators can induce the expression and activity of the other [55].

1.12. TGF-β–NOXs pathways

NOX enzymes are potent ROS generating enzymes. NOX2, NOX4 and NOX5 are important sources of H_2O_2, which are mainly regulated by innate immune system. However, some non-immune cells such as epithelial and bronchial cells in the lung are able to express some NOX enzymes [58]. These enzymes can be upregulated following apoptosis and senescence. These types of cell death are frequent in highly radiosensitive organs like bone marrow and intestine [59]. However, some others such as lung and brain have shown high incidence of senescence and upregulation of some subfamilies of NOXs [60]. In bone marrow, ionizing radiation causes death of progenitor and growing hematopoietic cells, mainly through apoptosis. After exposure to ionizing radiation, an increase in the level of TGF-β is observed in the bone marrow. Inhibition of TGF-β1 in mice following whole body irradiation has been shown to reduce the expressions of NOX2 and NOX4, leading to a reduction in oxidative stress and death of stem and progenitor cells [61].

1.13. TGF-β – COX-2 pathway

COX-2 is responsible for metabolism of prostaglandins. ROS is one of the second metabolites of this metabolism. TGF-β can augment the expression of COX-2 through non-canonical pathway. However, it has been confirmed that TGF-βRI can also induce COX-2 through canonical pathway [62]. Inhibition of COX-2 has been shown to reduce radiation injury in some tissues and organs such as joints, lung, bone marrow and skin [63]. The potent induction effect of TGF-β on COX-2 gene expression has been confirmed in bystander studies. This phenomenon will be discussed.

1.14. TGF-β–iNOS pathway

NO sources within tissues include some nitric oxide synthase (NOS) enzymes such as iNOS, neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) [64]. Among them, nNOS protects vessels against ROS. Low level of NO compared to ROS is responsible for vascular injury following exposure to ionizing radiation [65]. By contrast, upregulation of iNOS can augment oxidative stress and amplify toxic consequences of ionizing radiation. Although many types of cells are able to synthesize iNOS, it seems that activated macrophages are the main sources of NO following exposure to ionizing radiation. TGF-β is a potent inducer of iNOS. It has been confirmed that TGF-βRI–iNOS pathway plays a key role in NO production following exposure to ionizing radiation [66]. TGF-βRI–iNOS pathway plays a key role in bystander effect, which will be discussed.

1.15. TGF-β in radiation-induced bystander effect

Bystander effect is a phenomenon that causes the release of a wide range of agents from irradiated cells to induce some changes in other cells. As a result, bystander effect can augment radiation toxicity [67]. Irradiated cells amplify detrimental consequences of exposure to ionizing radiation via the release of several cytokines, microRNAs, nitric oxide (NO), exosomes *etc*. [68]. It seems that changes in the expressions of some ROS generating enzymes, mitochondria malfunction and changes in the methylation of some critical genes, play a key role in the bystander effect [67]. In vitro and in vivo studies have confirmed the role of TGF-β in oxidative stress and genomic instability in bystander cells/tissues. TGF-β can be released from macrophages following apoptosis or senescence of irradiated cells and then migrates and affect other areas. TGF-β can trigger macrophages to upregulate inducible nitric oxide synthase (iNOS), which lead to production of NO [66]. Inhibition of TGF-βRI can reduce the generation of NO in bystander cells. Furthermore, produced NO from direct irradiated cells can migrate to adjacent cells and attack the DNA [66]. NO can also interact

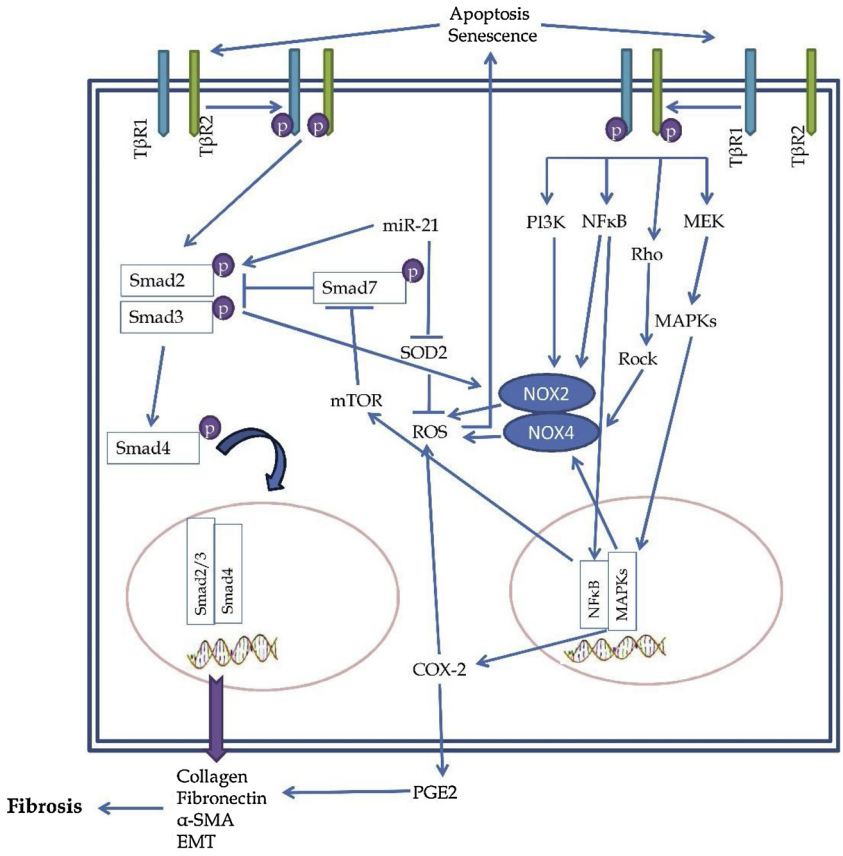

Fig. 3. Mechanisms of TGF-β induced fibrosis in irradiated organs. Apoptosis and senescence are the main causes of fibrosis following exposure to high doses of ionizing radiation. Both types of cell death mechanisms are able to trigger fibrosis through stimulation of canonical and non-canonical signaling pathways.

with generated superoxide (O_2) and hydrogen peroxidase (H_2O_2) from the mitochondria, leading to the production of higher half-life peroxynitrite free radicals.

One of the most important inducers of oxidative stress after exposure to radiation is upregulation or downregulation of epigenetic modulators. The role of epigenetic modulators such as miRNAs and siRNAs, and also changes in the global and promoter DNA methylation have been confirmed [67]. Some studies suggested that TGF-β can induce oxidative stress through upregulation of mir-21. In vitro evaluation of bystander mechanisms showed that MiR-21 can suppress activity of superoxide dismutase 2 (SOD2), leading to increased superoxide to antioxidant defense ratio [69]. Further studies showed that TGF-β is the most important stimulator of MiR-21 in bystander cells [70]. Usually, TNF-α can increase the activity of SOD2 and SOD3. However, it seems that in bystander cells, high expression of TGF-β receptors can suppress SOD2 and increase ROS level through TGF-β-mir-21-ROS pathway [53]. By contrast to MiR-21, MiR-663, an inhibitor of TGF-β, can reduce DNA damage and micronuclei formation in bystander cells. Interestingly, it has been shown that upregulation of TGF-βI in bystander cells results from suppression of MiR-663 following exposure of cells to ionizing radiation [71].

In vivo studies have shown that TGF-β can affect gene expression and oxidative stress in distant non-irradiated tissues. It has been shown that TGF-β upregulates the expression of COX-2 in lung tissues following local irradiation of the abdomen. Upregulation of COX-2 occurs following the expression of TGF-βRI and upregulation of both canonical and non-canonical signaling pathways [72]. Increased expression of TGF-βRI − COX-2 can increase ROS production, leading to mutations and oxidative injury in the DNA [73]. The upregulation of TGF-βRI − COX-2 has also been shown for heavy charged radiation particles [74]. As earlier mentioned, TGF-β can also increase the expression of NADPH oxidase enzymes. The significant increase in the expression of NOX2 and NOX4 subfamilies of NADPH oxidase have been observed for lung tissues following local irradiation of the pelvis [75].

It has been suggested that TGF-βRI–Smad2 pathway plays a critical role in the stimulation of micronuclei formation in bystander fibroblasts cells. Inhibition of TGF-βRI leads to suppression of micronuclei formation (a marker of DNA damage and probably ROS generation) in bystander fibroblast cells (Yin et al., 2015b). (Fig. 4) (Table 1)

1.16. TGF-β in tumor resistance to radiotherapy

Cancer cells and cancer stem cells (CSCs) are surrounded by immune system cells such as macrophages (including M1 and M2 types), dendritic cells, neutrophils, cancer associated fibroblasts (CAFs), T regulatory cells (Tregs) and cytotoxic CD8 + T lymphocytes (CTLs) [76]. The immune cells have several interactions that regulate suppression or progression of cancer cells' proliferation, stemness of CSCs, angiogenesis and finally tumor growth. TGF-β is one of the most important regulators of tumor growth via regulation of several functions within the tumor [77]. Although TGF-β may have a suppressive effect in the proliferation of cancer cells in grade I tumors, it can suppress immune activation and facilitate immune escape of cancer cells [11]. The expression of TGF-β has a direct relation with tumor grade, which indicates its critical role in tumor growth [78]. Radiation is a potent inducer of TGF-β within TME. Although radiation kills cancer cells through different mechanisms, dying cells trigger the release of several cytokines including TGF-β to promote cancer progression and reverse depletion of cells after radiotherapy [11]. In this section, we explain the cellular and molecular mechanisms of TGF-β role in tumor resistance to radiotherapy.

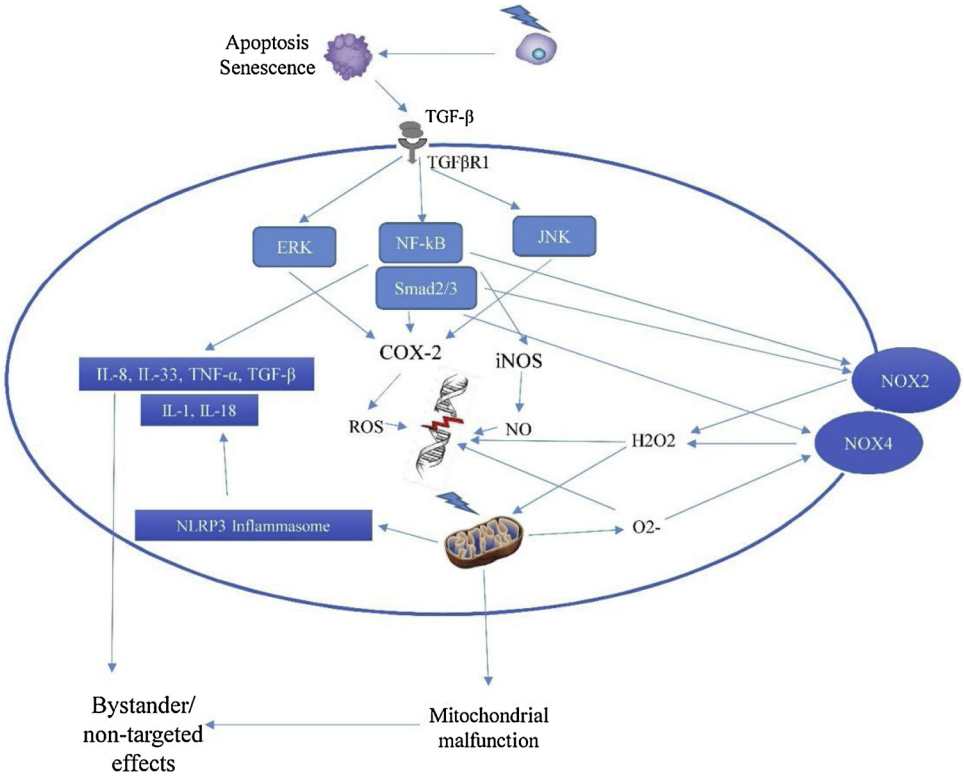

Fig. 4. Mechanisms of TGF-β induced oxidative stress in directly irradiated and bystander cells/tissues. TGF-β can induce ROS/NO generation in both immune and non-immune cells. Upregulation of COX-2, iNOS, NOX2&4, and also mitochondrial malfunction are main reasons for chronic oxidative stress in both direct irradiated and bystander cells/organs.

1.17. TGF-β suppresses immune system and facilitates cancer immune escape

The balance between pro-tumor and anti-tumor immune cells plays a key role in the fate of cancer therapy. CTLs, M1 macrophages and NK cells are the most important anti-cancer cells within TME. On the other hand, Tregs, CAFs and M2 macrophages increase proliferation and viability of cancer cells and CSCs [76]. TGF-β plays a central role in the regulation of cancer and CSCs proliferation and suppression of anti-cancer cells including CTLs and NK cells [11]. CAFs are the main source of TGF-β within TME. However, Tregs plays a key role in suppression of CTLs and NK cells via release of TGF-β. Release of TGF-β also shifts polarization of macrophages to M2 type. The positive feedbacks between these cells potentiate proliferation of cancer cells and promote stemness of CSCs [76].

As mentioned earlier, ionizing radiation can kill cancer cells through different mechanisms. Immunogenic cell death triggers lymphocytes to release inflammatory cytokines such as IFN-γ and TNF-α to kill cancer cells [76]. However, overexpression of some inflammatory mediators like NFκB can increase survival of cancer cells via induction of anti-apoptotic genes [16]. Thus, inflammatory responses may be in favor of elimination of cancer cells. Tolerogenic cell death including apoptosis and senescence trigger the release of TGF-β and IL-10, which suppress inflammation within TME. Apoptosis to necrosis/necroptosis ratio is highly dependent on tumor type and dose of ionizing radiation. In some cancers such as bone marrow derived malignancies, apoptosis is the most common type of death in irradiated cells [23]. TGF-β can suppress inflammatory responses and immune activity against cancer cells through different mechanisms. TGF-β inhibits the release of IL-2 to prevent proliferation of CTLs and NK cells [11]. TGF-β also facilitates proliferation of CD4+ to Tregs and fibroblasts to CAFs. It also shifts differentiation of macrophages into M2 cells. These cells trigger further release of anti-inflammatory cytokines including TGF-β, IL-4 and IL-13, which are associated with further suppression of CTLs and NK cells. TGF-β through this way inhibits secretion of anti-cancer cytokines including IFN-γ and TNF-α [76]. Thus, inhibition of TGF-β can be suggested for reducing exhaustion of immune system and to reverse infiltration of tumor promoting cells. TGF-β suppression in combination with radiotherapy has also been shown to increase the numbers of CTLs and NK cells within tumor [10].

Immune checkpoints are important targets that are interesting for immunotherapy. Targeting immune checkpoints with immune checkpoint inhibitors (ICIs) in combination with radiotherapy has shown promising results [76]. PD-1 and CTLA-4 are two common immune checkpoints that are used to enhance immune activity against cancer progression. These proteins mediate apoptosis of CD4+, CTLs and DCs, thus reduce immune system's activity within TME [79]. Unfortunately, the sensitivities of these cells are higher compared to Tregs and CAFs. This leads to higher ratio of tumor promoting cancer cells compared to anti-tumor cells. Reversing this ratio is critical for increasing tumor response to radiotherapy [76]. TGF-β plays a key role in the regulation of PD-1 and CTLA-4, thus promotes apoptosis in CTLs and DCs [80]. TGF-β has a key role in the regulation of T cell receptor (TCR) activation, which lead to upregulation of PD-1 and induction of apoptosis in CD8+ cytotoxic lymphocytes [81]. Proliferation of Tregs by TGF-β can also increase the production of both PD-1 and CTLA-4 within TME [82]. (Fig. 5)

1.18. TGF-β in DNA damage response (DDR) in cancer cells

TGF-β has a close relation with DDR and adaptation to clastogenic agents such as ionizing radiation [83]. It seems that TGF-β has a role in phosphorylation of DNA repair enzymes. Thus, targeting of TGF-β has been proposed for induction of apoptosis and increasing the therapeutic efficiencies of a wide range of anti-cancer modalities. Suppression of TGF-β has been shown to reduce phosphorylation of H2AX, ATM and p53, critical enzymes for initiation of DDR following exposure to radiation [83]. As TGF-β has a multi-effect cytokine, it may have different effects on various types of cancer in this way. Treatment of human nasopharyngeal carcinoma CNE-2 cell with TGF-β has been shown to induce apoptosis, while activation of Smad3, a downstream of TGF-β increases cell resistance [84]. However, it has been confirmed that it

Table 1
Results of targeting of TGF-β signaling for normal tissue sparing.

Route	Cells/tissues	Inhibitor	Findings	References
Mice	Bone marrow	SB431542	Targeting TGF-β causes attenuation of NOX2 and NOX4 expressions, and reduces ROS production.	[61]
In vitro	Mice prostate fibroblasts	–	Inhibition of senescence and NOX4 reduce the expression of canonical pathway of TGF-β and attenuate the level of Smad2-4.	[51]
In vitro	Fibroblast cells co-cultured with HaCaT keratinocytes	SB431542	Inhibition of TGF-βRI leads to prevention of micronuclei formation and phosphorylation of Smad2 in bystander cells.	(Yin et al., 2015b)
In vitro	Hela cells	MiR-663	Targeting TGF-βRI by MiR-663 reverses bystander effect.	[71]
Mice	Lung	Statins and Y-27632	Selective inhibition of Rho/Rock pathway attenuates late fibrosis in mice lung.	[43]
Rats	Intestine	Pravastatin	Pravastatin can reduce accumulation of collagen and fibronectin through suppression of Rho/Rock pathway.	[47]
Mice	Lung	–	Knockdown of Syndecan-2 can induce the upregulation of TGF-β – Rho/Rock pathway.	[46]
Mice	Lung	Lovastatin and EHT1864	Inhibition of Rho reduces DNA damage and apoptosis, thus may attenuate late fibrosis.	[49]

increases resistance of a wide range of cancers. Molecular analyses show that TGF-β inhibits regulation of miR-182, which itself causes suppression of BRCA1 and forkhead box O3 (FOXO3). Thus, suppression of TGF-β can downregulate FOXO3 and BRCA1, thereby preventing phosphorylation of ATM and attenuation of the homologous recombination (HR) repair pathway [85]. Attenuation of these enzymes following suppression of TGF-β has been shown to reduce resistance of cancer cells to ionizing radiation [86]. In the absence of activated ATM and HR, cells use other alternative DNA repair pathways that are error prone and higher cell death probability [85]. (Fig. 6)

1.19. TGF-β interactions with hypoxia and angiogenesis during radiotherapy

Angiogenesis is the formation of new vessels, which is a common mechanism during growth of embryo and also tissues in adults. Angiogenesis in tumors is a critical demand for tumor growth [87]. In fact, tumors progress faster than their vessels, however, this causes chronic hypoxia and limits sufficient supply of nutrients for cancer cells [88]. Insufficient nutrient can change the metabolism of cancer cells, however, hypoxia can trigger angiogenesis through stimulation of some genes such as HIF-1 and VEGF [89]. Hypoxia is a potent inducer of TGF-β. Hypoxia regulates macrophages to M2 type cells, which are a source of TGF-β [11]. Furthermore, CAFs within hypoxia condition release TGF-β to promote angiogenesis [11]. In irradiated glioma, it has been shown that hypoxia induces homing hematopoietic stem cells towards tumor via TGF-β-HIF-1 pathway [90]. Irradiation of cancer cells has been shown to increase the release of TGF-β and the expressions of TGF-βI and TGF-βII [91]. There are limited studies on the interactions between angiogenesis and TGF-β in irradiated tumor or cancer cells. These interactions lead to tumor resistance through several mechanisms. (Fig. 6) Blockade of TGF-βI has been shown to reduce microvessel density in irradiated glioblastoma [92]. Although it has been observed that inhibition of TGF-β can sensitize cancer cells to ionizing radiation and suppress tumor growth [34,93], the exact interactions between TGF-β and angiogenesis factors during radiotherapy need to be elucidated.

1.20. TGF-β targeting and EMT in radiotherapy

EMT within TME is a process in which the epithelial cells undergo some genetic changes to become mesenchymal stem cells. These new cells have ability to undergo differentiation to various types of cells. EMT and also polarization of mesenchymal cells to other types of cells is highly dependent on releases and interactions within TME. It has been shown that irradiation can induce EMT and stemness of cancer cells, which leads to radioresistance of cancer cells [94]. However, inhibition of TGF-βRI can reverse radioresistance of irradiated cells [91]. TGF-β plays a key role in EMT and also polarization of mesenchymal cells to tumor promoting cells such as CAFs [95]. Radiation-induced EMT is also mediated through polarization of macrophages into M2 cells, a process which is mediated by TGF-β signaling [96]. In A549 cells, up-regulation of MiR-3591-5p plays a key role in the activation of TGF-β-Smad2/3 pathways and EMT following irradiation. Inhibition of this pathway has also been shown to suppress phosphorylation of Smad2/3 and EMT [36]. Similar results have been reported for lung cancer xerografts [97].

1.21. Clinical trials for TGF-β blockade in combination with radiotherapy

Due to several positive results from experimental studies, some clinical studies have been designed to use TGF-β antagonists for cancer patients undergoing radiotherapy. In the first published clinical study, patients with metastatic breast cancer were treated with TGF-β blocking antibody fresolimumab and received focal radiotherapy to metastatic sites. Results showed interesting increase in overall survival,

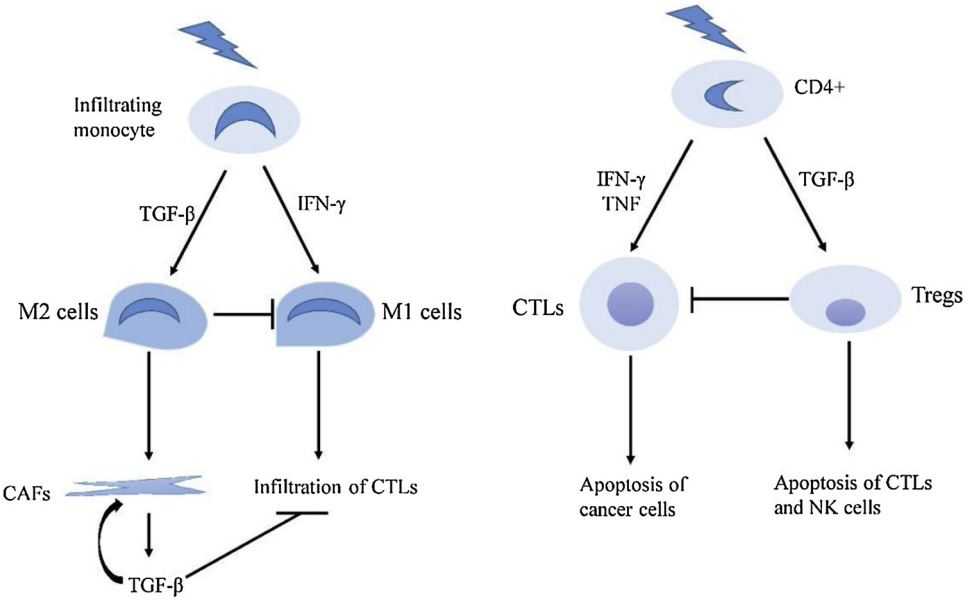

Fig. 5. Mechanisms of TGF-β induced immune system suppression and immune escape of cancer (stem) cells. TGF-β induces infiltration of monocytes and polarization into M2 type of macrophages. On the other hand, TGF-β plays a key role in the polarization of fibroblasts into CAFs and CD4 + T cells into Tregs. These cells are able to potentiate each other through a positive feedback loop. TGF-β is a key mediator of this positive feedback.

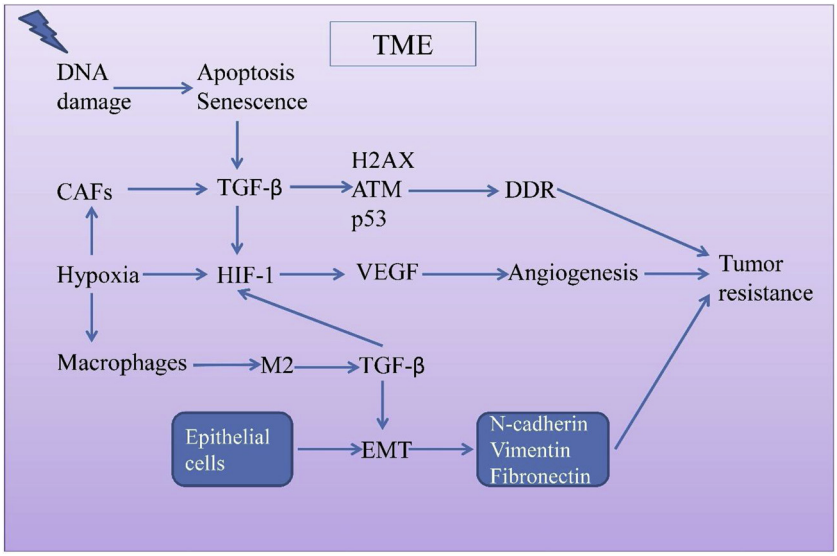

Fig. 6. TGF-β-induced tumor resistance following radiotherapy. Apoptosis and senescence after radiotherapy trigger release of TGF-β by macrophages. However, hypoxia can further amplify release of it. TGF-β can increase tumor resistance to radiotherapy through various mechanisms including stimulation of DDR, EMT, and angiogenesis.

which was associated with increased numbers of peripheral CD8 + T cells [98]. Results of this study confirmed experimental outcomes of TGF-β inhibition in tumor, which can boost immune system against cancer. (Table 2)

Some other clinical trials are ongoing and are close to completion. Another clinical trial has attempted to use anti-TGF-β LY2157299 for metastatic breast cancer patients (NCT02538471). Fresolimumab as anti-TGF-βI is also being used in combination with stereotactic ablative radiotherapy (SAR) for patients with non-small cell lung cancer (NCT02581787). A study of LY2157299 combination with radiation and chemotherapy with Temozolomide for patients with grade 3–4 glioma tumors has been completed (LY2157299) and another trial for rectal cancer patients is ongoing (NCT02688712).

2. Conclusion

As mentioned in this paper, TGF-β plays a key role in radiotherapy outcomes. Due to the critical role of TGF-β in both tumor resistance and normal tissue injury, it affects therapeutic efficiency potently. Fibrosis is probably the most common effect of TGF-β in normal tissues. Fibrosis is a very important side effect of radiotherapy that may affect patients' quality of life or could lead to death. TGF-β triggers the deposition of collagen and fibronectin through various signaling pathways including Rho/Rock, Smad2/3, NADPH oxidase, COX-2, epigenetic modifications and also hormone changes including renin-angiotensin system. TGF-β also stimulates the activities and regulation of some pro-oxidant enzymes including iNOS and especially NOX2 and NOX4, which lead to continuous production of free radicals. It has been confirmed that these changes are associated with genomic instability, which is a hallmark of carcinogenesis.

TGF-β in TME is the most potent suppressor of immune system's activity against cancer cells. TGF-β can directly promote cancer cells' proliferation. Furthermore, it induces proliferation of Tregs and CAFs, which are sources of TGF-β in TME. The positive feedback between TGF-β, Tregs and CAFs suppresses proliferation of CD8 + T cells and NK cells. Furthermore, TGF-β inhibits the release of anti-cancer cytokines such as IFN-γ and TNF-α from CD8 + T cells. Stimulation of DDR in response to radiotherapy also increases resistance of cancer cells. TGF-β can trigger angiogenesis and metastasis via stimulation of EMT and HIF-1. Targeting of TGF-β in experimental studies has shown promising results for both normal tissues and tumor. The combination of anti-TGF-β drugs with radiotherapy may enhance tumor response and

Table 2
Summary results of TGF-β signaling targeting in combination with radiotherapy for cancer cells.

References	Findings	Drug/inhibitor	Cell/tissue	Route
[92]	Selective targeting of TGF-βI reduces angiogenesis, increases apoptosis and suppresses EMT and stemness.	LY2109761	Glioblastoma	In vitro/ in vivo
[84]	Upregulation of Smad3 is associated with resistance to radiation, while TGF-βI upregulation can induce apoptosis.	The Smad3 overexpression vector (LV-Smad3)	Human nasopharyngeal carcinoma GNE-2 cell	In vitro/ in vivo
[83]	Inhibition of TGF-βI prevents phosphorylation of γH2AX and P53, leading to inhibition of DDR.	LY364947 and LY2109761	MDA-MB-231	In vitro
[86]	Inhibition of TGF-β attenuates the activities of DNA repair enzymes such as p53 and ATM, leading to further DNA damage and cancer cell death.	LY364947	A549, NSCLC NCI-H1299, and NCI-H292	In vitro/ in vivo
[97]	Suppression of TGF-βI prevents EMT markers including N-cadherin and Vimentin.	SB431542 or halofuginone	Lewis lung carcinoma (LLC) xenograft	In vivo
[98]	An increase in overall survival and increase in peripheral CD8 + as well as blood mononuclear cells were observed. No remarkable increase in CD4+ counts was reported.	Fresolimumab	Metastatic breast cancers	Clinical trial

also reduce side effects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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