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The Radiobiology of TGFβ

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Abbreviations: Transforming growth factor β, TGFβ; radiation therapy, RT; tumor microenvironment, TME; latency associated peptide, LAP; DNA damage response, DDR; ataxia-telangiectasia mutated, ATM; glioblastoma multiforme, GBM; head and neck squamous cell carcinoma, HNSC; human papilloma virus, HPV; homologous recombination, HR; non-homologous end-joining, NHEJ; alternative end-joining, alt-EJ; polymerase theta, POLθ; poly(ADP-ribose) polymerase 1, PARP; programmed cell death-ligand (PD-L1)

Abstract

Ionizing radiation is a pillar of cancer therapy that is deployed in more than half of all malignancies. The therapeutic effect of radiation is attributed to induction of DNA damage that kills cancers cells, but radiation also affects signaling that alters the composition of the tumor microenvironment by activating

transforming growth factor β (TGFβ). TGFβ is a ubiquitously expressed cytokine that acts as biological lynchpin to orchestrate phenotypes, the stroma, and immunity in normal tissue; these activities are subverted in cancer to promote malignancy, a permissive tumor microenvironment and immune evasion. The radiobiology of TGFβ unifies targets at the forefront of oncology—the DNA damage response and immunotherapy. The cancer cell intrinsic and extrinsic network of TGFβ responses in the irradiated tumor form a barrier to both genotoxic treatments and immunotherapy response. Here, we focus on the mechanisms by which radiation induces TGFβ activation, how TGFβ regulates DNA repair, and the dynamic regulation of the tumor immune microenvironment that together oppose effective cancer therapy. Strategies to inhibit TGFβ exploit fundamental radiobiology that may be the missing link to deploying TGFβ inhibitors for optimal patient benefit from cancer treatment.

RADIATION ONCOLOGY

The physical properties of radiation that cause DNA damage have been elegantly harnessed to control cancer growth in radiation oncology (RO). However, tumor control is not uniform, reflecting not only the heterogeneity of disease, but also incomplete understanding of the underlying radiobiology of response. Radiobiology is described in terms of the five Rs—repair, repopulation, redistribution, reoxygenation, and radiosensitivity [1]—that capture decades of experimental studies to inform radiation oncology practice. Indeed, the paradigm of biologic effect beginning with radiation-induced DNA damage paved the way to systems biology by integrating the effects of biological mechanisms over scales of organization (e.g., DNA repair, cell cycle distribution, radiosensitivity) and time (e.g., repopulation, redistribution, reoxygenation) in designing regimens of radiation therapy (RT) in cancer [2, 3].

Radiation oncology has made major strides in harnessing the physical properties of ionizing radiation to deliver accurate and precise therapy to patients, improving outcomes mostly through improved physical targeting of tumors while sparing normal tissues over the last decade [4]. However, the considerable enhancements made to radiation dose delivery have not been equally matched by translating the deep molecular understanding of how diverse cells respond to radiation. To promote precision medicine in RO, we need to not only physically target the tumor but also take aim at the biological mechanisms that can be exploited to augment tumor control by amplifying radiation effects that can promote anti-tumor immunity. Mechanisms of resistance can also be the basis to identify patients who will likely fail therapy; treatment with agents that overcome resistance thereby biologically augment RT.

Here we focus on radiation-induced stress signaling that mediates crosstalk between cancer, the tumor microenvironment (TME) and immune evasion that have yet to be fully investigated in patients or integrated into treatment strategies. The TME is a key source of signals that range from extracellular growth factors and cytokines to membrane-bound receptors to insoluble extracellular matrix that together impact tumor control and normal tissue toxicities [5]. Indeed, the irradiated TME can ultimately determine cancer response or resistance [6-10], but the lack of a detailed roadmap to exploit this vulnerability is a gap that hampers the optimal use of RT.

The next generation of radiobiology will be understanding how the *remodeling* of the TME mediates tumor control and, ideally, promotes immune *rejection*. These 'new' Rs emphasize the importance of intra- and extracellular signaling, and the interaction between different cell types as potential targets of multi-modal therapies. Together, the physiological context, intercellular communication within tumors and tissue, as well as systemic signaling provide important targets to manipulate to improve response to RT.

Transforming growth factor β (TGFβ) is arguably the most prominent factor in irradiated tissues. TGFβ is a widely expressed cytokine that sequestered in the TME in a latent form, but once activated by release it is extraordinarily potent and pleiotropic [11]. Radiation elicits rapid and persistent TGFβ activation. TGFβ is one of the most intensively studied oncology targets because it is:

- **Ubiquitous**: all cancers produce and activate TGFβ.
- **Pleiotropic**: TGFβ regulates manifold pro-tumorigenic phenotypes, including TME composition, invasiveness, immunosuppressive immune cells, cancer-associated fibroblasts, and vascular remodeling.
- **Central**: TGFβ is a hub of intracellular signaling that orchestrates proliferation, self-renewal, cell fate, and genomic integrity, as well as mediating intercellular signaling between of cells, tissues, and organs.

TGFβ is classically defined as a tumor suppressor early in carcinogenesis yet paradoxically works as a tumor promoter in established cancers. TGFβ is a lynchpin that links diverse cell phenotypes and tissue

function during development, homeostasis, and response to injury. These critical processes are appropriated during carcinogenesis to promote malignancy, restructure the TME, and evade anti-tumor immunity. The paradox of how cells convert from exquisitely sensitive to impervious to growth control is only one part of the TGFβ conundrum; its action in the TME and immunity are also puzzling when viewed from the perspective of cancer therapy. How does one target a lynchpin without everything becoming unraveled?

Unlike other targeted pathways, TGFβ is not a tumor-driver, as it does not promote survival *per se* or proliferation that contribute to tumor growth. Rather, it drives the malignant composition of the tissue in which the tumor resides, the TME [12]. Although the idea that TME impacts cancer response or resistance is conceptually strong [6-10], it has nonetheless proven to be a challenge to position a therapy that targets the soil rather than the seed. Indeed, the lack of a detailed roadmap to exploit this vulnerability is a gap underscored by the absence of clinical approval for the small molecules, proteins and nucleic acid-based agents that neutralize or block TGFβ [12, 13]. Here I will review studies from my laboratory and others showing that RT provides a definitive setting for advancing use of TGFβ inhibitors to benefit cancer patients.

TGFβ Biology: A primer

Three genes, *TGFB1*, *TGFB2* and *TGFB3*, encode the mammalian isoforms. The synthesis of protein for each is similar to that of the founding member, *TGFB1.* Latent TGFβ1 consists of a 24kD homodimer that is non-covalently associated with its latency associated peptide (LAP), a ~80kD N-terminal pro-peptide dimer that contains the signal sequence for secretion [14, 15]. Together, TGFβ and its corresponding LAP constitute the ~125kD small latent complex, in which latency, i.e., blocking receptor binding, is conferred by TGFβ's association with LAP. Latent TGFβ binding proteins, of which there are four, can covalently complex with LAP via a disulfide bond, forming the large latent complex that is subsequently secreted and sequestered in the extracellular matrix [16]. Latent TGFβ binding proteins are necessary for efficient assembly and secretion of the complex, and enable latent TGFβ to be sequestered extracellularly [17, 18].

Broadly, all mammalian cells produce latent TGFβs, which are abundant in the extracellular matrix and in circulation, and all cells have receptors. Thus, TGFβ bioactivity is governed by a process called activation that involves the extracellular release of the mature cytokine from LAP. In the lab, exogenous TGFβ activation can be elicited by acidic pH, high (>-80° C) temperature and even mechanical dissociation [19, 20]. Physiological activation of TGFβ can occur through various mechanisms that include proteolytic cleavage (e.g. plasmin, furin), force-mediated activation via interactions with integrins and other cell membrane proteins [21], and exposure to reactive oxygen species [22]. Although all three mammalian TGFβ isoforms generate ligands that can bind the same receptors, the evidence of rapid TGFβ activation occurring in irradiated tissues led to the discovery that efficient activation of TGFβ1 can be elicited by exposure of the latent protein to reactive oxygen species in solution [23]. This mechanism is restricted to the protein product of *TGFB1* due to a redox-sensitive methionine in its LAP. Thus, latent TGFβ1 is an exquisitely sensitive sensor of oxidative stress outside the cell, which endows it with the capacity to orchestrate multicellular processes.

Once active TGFβ is released from the latent complex, any of the three mammalian TGFβ ligands can bind TGFβ type 1 and II receptors, TBRI and TBRII, which are serine-threonine kinases. Ligand binding brings the receptors together so that constitutively active type II receptor recruits and phosphorylates the type I receptor, that in turn phosphorylates the so-called, receptor SMADs, SMAD 2 and 3. Phosphorylated SMAD 2 and 3 complex with SMAD4 that act together in the nucleus to form transcription complexes that mediate transcription of TGFβ target genes. Nuclear localization of phosphorylated receptor SMAD, which is often used as an index of TGFβ activity, is transient due to receptor trafficking and degradation [24]. Keeping in mind that the mammalian TGF-β family consists of 33 members that signal via cell surface

receptors to elicit canonical signaling via the SMAD complexes, there is also additional complexity from non-canonical signaling and inhibitors that act at several points (reviewed in [25].

TGFβ is often described as a canonical tumor suppressor. Consistent with this, escape from TGFβ growth regulation is a requisite for malignancy. Loss of response to TGFβ as a growth inhibitor and increased expression of TGFβ has been associated with malignant conversion and progression in breast, gastric, endometrial, ovarian, and cervical cancers, as well as glioblastoma and melanoma [26]. Inactivation of the *SMAD4* gene through homozygous deletion or intragenic mutation occurs frequently in association with malignant progression in pancreatic and colorectal cancer [27]. However, mutation of the TGFβ pathway occurs only occasionally in most other human cancers. In a study of more than 500 breast cancers, Reiss and colleagues showed that 92% were positive for nuclear, phosphorylated SMAD2, indicating activation of the TGFβ pathway [28]. Indeed, many TGFβ transcriptional responses are intact while cancer cells have escaped the control of proliferation. More importantly, it is clear that increased TGFβ in cancer can act in a variety of ways to promote neoplastic progression. Production of TGFβ by malignant cells acts on the host to suppress antitumor immune responses, to enhance extracellular matrix production and to augment angiogenesis (reviewed in [29]). These activities resemble those induced by TGFβ during wound healing and may create a 'permissive' microenvironment that promotes malignant growth by acting on the host.

Genomic instability is a less well-recognized consequence of TGFβ loss, yet deletion of *Tgfb1* greatly increases genomic instability in murine epithelial cells [30]. Using cultured keratinocytes isolated from newborn *Tgfb1* null, heterozygote and wildtype mice, Yuspa and colleagues showed that *Tgfb1* null cells spontaneously immortalized more readily than TGFβ competent cells. Compared to wildtype cells, *Tgfb1* null cells gave rise to 1000-fold more mutant clones resistant to PALA, an event requiring amplification of the dihydrofolate reductase gene. This unexpected phenotype was difficult to place within the pathways known to be controlled by TGFβ. Following up on this finding, our lab found increased centrosome aberrations, chromosomal instability and spontaneous DNA damage in nonmalignant human epithelial cells in which TGFβ signaling was inhibited by a small molecule inhibitor of the TGFβ type I receptor kinase [31]. We also showed that *Tgfb1* heterozygote mammary epithelium, which express only 10-30% of wild type protein levels, exhibits genomic instability at a level comparable to *Trp53* heterozygote epithelium.

This observation gained more importance with the finding that TGFβ regulates the expression of key DNA damage response (DDR) proteins [32], and our study showing that epithelial tissues of *Tgfb1* null embryos fail to undergo apoptosis or cell cycle arrest in response to high dose (5 Gy) radiation [33]. Our subsequent study found that faulty cell fate decisions could be attributed to 70% reduction of ATM (ataxia-telangiectasia mutated) kinase activity, which broadly compromises the DDR [34]. ATM is a phosphoinositide 3-kinase related serine/threonine kinase that mediates DNA damage responses to initiate, recruit and activate a complex program of checkpoints for cell cycle, apoptosis, and genomic integrity. Mutations in human ATM lead to ataxia-telangiectasia, which is characterized by cellular radiosensitivity, in which cells also have high levels of chromosome aberrations [35]. Induction of ATM kinase activity in response to double-strand breaks in turn phosphorylates numerous substrates, thereby modulating cell fate decisions. ATM controls DDR effectors, each of which activate transducers to control cell fate [36].

TGFβ regulates ATM kinase activity (see Figure 3 and related text below; refs [34, 37]). Both *Tgfb1* genetic knockout in mouse cells and TGFβ signaling inhibition in human cells decrease radiation-induced ATM kinase activity and hence, autophosphorylation, leading to reduced phosphorylation of critical DNA damage transducers, abrogation of the cell cycle block and increased radiosensitivity [33, 34]. The ability of exogenous TGFβ to restore these responses indicates that this is both cell intrinsic and distal to TGFβ signaling. Our studies also showed that inhibiting TGFβ signaling in irradiated human cells phenocopies

the molecular and cellular consequences of genetic deletion. One of the most striking phenotypes of A-T cells is extreme cellular radiosensitivity demonstrated in clonogenic survival assays. Consistent with reduced ATM activity, *Tgfb1* genetic deletion in murine cells or pathway inhibition in human cells increases radiosensitivity. Phosphorylation by ATM of key effectors, most prominently p53, determine how cells respond DNA damage—whether to repair, cell cycle arrest, die, senesce, or proliferate is orchestrated via a complex network of phosphorylations [38].

Cells maintain genomic stability in the face of relentless challenges by environmental stresses that induce DNA breaks and activate DNA damage pathways mediated by ATM and its downstream effectors that lead to damage-induced cell-cycle checkpoints and DNA repair [38]. Recognition of the role of TGFβ in DDR led us to consider the consequences of compromised TGFβ signaling Centrosome aberrations and tetraploidy increase in *Tgfb1* heterozygote mammary epithelia as a function of age; inhibiting TGFβ signaling in human epithelial cells increased centrosome aberrations, aneuploidy, tetraploidy and spontaneous DNA damage, all measures of genomic instability [31], recapitulating what was shown by Glick more than a decade before, that *Tgfb1* null keratinocytes are highly genomically unstable[30].

Women with "low signaling" TGFβ genetic polymorphisms have a 2-fold increased risk for breast cancer [39, 40]. *Tgfb1* heterozygote mice stressed with oncogene expression or chemical carcinogen exposure, exhibit increased tumor incidence and size [41] and decreased tumor latency [42, 43]. Moreover, genomic instability is dramatically increased in *Tgfb1* null murine cells [30, 44]. Thus, attenuated TGFβ signaling may amplify the possibility of neoplastic transformation. Early malignant lesions of the breast accumulate genomic damage, evidenced by abnormal centrosomes, chromosome instability and activation of the DNA damage response, often without p53 mutations [45, 46]. All cancer cells also have some degree of genomic instability, and acquisition of genomic instability by normal cells dramatically increases the likelihood of malignant transformation [46].

The conundrum of why tumors maintain TGFβ expression and signaling when it is an extremely potent growth inhibitor gains clarity when control of the genomic integrity is incorporated. All cancers escape TGFβ growth regulation yet maintain elevated levels of TGFβ activity that promote tumor progression seems paradoxical. But cancer cells that have high genomic instability fail to progress; indeed, invasive breast tumors are more genomically stable than ductal carcinoma in situ [47]. We speculate that the necessity for TGFβ signaling to maintain genomic integrity suggests that TGFβ acts to suppress cancer by both ensuring genomic integrity as well as growth control. During malignant progression, loss of growth control is a prerequisite, but complete loss of genomic integrity would be lethal. Hence cancers manage to dissociate these tumor suppressive pathways, which protects malignant cells by limiting the level of genomic instability. As discussed below, this bifurcation also enables recovery from DNA damage induced by radiation and other therapies.

TGFβ Biology in Radiation Therapy

Radiation induces TGFβ activity in vitro and in vivo both in normal tissues and cancer [48-54]. We and others have shown that TGFβ mediates the response to radiation [55, 56], but it is clear that targeting TGFβ not only changes tumor cell biology but also affects the tumor-promoting TME composition, cancer-associated fibroblast phenotypes, bone marrow derived cell recruitment, ECM remodeling, and angiogenesis [57-60]. Moreover, the production of TGFβ by tumor cells is key to the systemic immune response [13]. Clearly exogenous means of inhibition, like neutralizing antibodies and small molecule inhibitors that can be used in the clinic, can affect tumor cells, the TME and immunity. Thus, treatment with extrinsic TGFβ inhibitors simultaneously modulates cancer cells, TME, host cells and systemic immunity (**Figure 1**).

Mouse models of breast cancer illustrate the therapeutic potential of RT and TGFβ blockade. The addition of TGFβ neutralizing antibodies to radiation increases survival in two breast cancer models, 4T1 and TS/A, and showed an additive survival benefit in a triple combination with anti-programmed cell death-ligand (PD-L1) [56]. DNA damage repair deficits represents an exploitable vulnerability in cancer [61], which are often empirically revealed by clinical trials, e.g., use of cisplatin in head and neck squamous cell carcinomas or nitrosoureas in glioblastoma. Recognition that the nature of specific DNA damage repair deficits can be the basis for selection of treatment led to the concept of synthetic lethality [62]. The canonical example is synthetic lethality from poly(ADP-ribose) polymerase 1 (PARP) inhibitors in the context of homologous recombination (HR) deficits originally defined for germline *BRCA* mutations in breast and ovarian cancer patients. The utility of PARP inhibitors has been expanded to cancers with somatic mutations in *BRCA1*/2, or other HR components, defined as HR deficit phenotype, but patients with HR deficient tumors also respond well to standard of care interstrand crosslinking platinum therapies [63].

Glioblastoma (GBM) is characterized by a high degree of radioresistance evidenced by inevitable local and/or disseminated recurrence. Several clinical trials are underway combining TGFβ inhibition with cancer RT and chemotherapy, including a phase II trial in glioblastoma. Addition of TGFβ inhibitors improves radiation response in preclinical models of GBM [64, 65]. Zhang et al. specifically reported that the addition of the small molecule inhibitor of TGFβ receptor type I and II kinase, LY2109761, to the current standard of care treatment (radiation and the oral alkylating agent temozolomide) provided benefit. In addition to radiosensitization and tumor growth delay, TGFβ signaling blockade had anti-angiogenic and anti-migration effects as well. Mengxian et al. similarly reported radiosensitization, tumor growth delay, and improved survival with the addition of the same small molecule inhibitor of TGFβ, LY2109761, without combining with temozolomide. They further demonstrated that either TGFβ inhibition or radiation decreased self-renewal of glioma stem-like cells in a neurosphere assay, and a greater decrease when these were combined. Our studies added significantly to the growing body of evidence that TGFβ is a therapeutic target in GBM [66]. First, we showed that autocrine TGFβ potentiates an effective molecular DNA damage response and that radiation-induced TGFβ mediates self-renewal signals in glioma initiating cells. Second, the magnitude of radiosensitization (dose enhancement ratio (DER) ~1.25 by clonogenic assay) is similar to that shown by treated with temozolomide (DER 1.32) [67]. Considering that the addition of temozolomide to radiation therapy in the treatment of GBM was one of the largest breakthroughs in this disease in decades and is now standard of care, radiosensitization of this magnitude reported here must be considered significant, particularly since the radiation sensitivity of glioma initiating cells increased nearly 3-fold. Lastly, we found that glioma initiating cells produce more TGFβ, which improves effective execution of the DDR and increases survival following therapy [66].

These data all demonstrate that radiation elicits TGFβ activity but the challenge of TGFβ is its pleiotropy—what is TGFβ doing in a particular context? In addition to radiation, other genotoxic cancer therapies, e.g., cisplatin and temozolomide, elicit TGFβ activity that persists after completion of treatment (reviewed in [68, 69]). As most cancer patients have been or will be treated with RT, understanding RT effects is critical for optimizing benefit [70, 71]. Major clinical trials combine classes of immunotherapy, chemotherapy, targeted therapy and/or RT, but the number of possible combinations that could be tested far exceeds the resources and patient populations available [72, 73]. Hence understanding how TGFβ affects the irradiated tumor is critical to identifying the right combination for a specific patient

Immunotherapy, RT and TGFβ

A primary function of TGFβ in the immune system is to control auto-immunity, which is subverted in malignancy to anti-tumor immunity [11]. For immunoncology (IO), drugs target the mechanisms by which tumors suppress immune rejection, the type, the density and the location of immune cells within human

tumors are important factors that affect prognosis [74]. TGFβ activity promotes a permissive TME and facilitates immune evasion. Different populations of lymphocytes commonly infiltrate tumors: their distribution and the prevalence of one subset over the other has been shown to be associated with outcome, both preclinically and clinically. For instance, the type, density, and location of immune cells found within several hundred human colorectal tumor specimens was a better predictor of survival than the standard histopathology used to stage cancer. These results were validated in two additional patient populations [75] and similar patterns were found in other human tumor sites [76, 77]. In breast, the extent of lymphocyte infiltration in pre-treatment biopsies was found to be a significant independent parameter that predicts pathologic complete response to regimens of anthracycline/taxane-based chemotherapy in multivariable regression analysis that included standard predictive clinical-pathologic factors [78].

Ionizing radiation is the canonical example of a DNA damage agent whose benefit is ascribed to cell kill in the irradiated field, yet a range of studies have showed that radiation in conjunction with IO can promote systemic immune response [79-81]. Formenti and Demaria conceptualization of radiation as an 'in situ vaccination' has since been demonstrated in the clinic [82] and additional research has defined mechanisms by which radiation can synergize with IO [83-85].

Given that durable response to IO like checkpoint inhibitors is achieved in less than 50% of patients, considerable effort has focused on who will likely respond and why. A particularly compelling study identified the association between response to IO and the mismatch repair (MMR) or microsatellite instability phenotype in colon cancer patients [86]. MMR is exhibited by 20% of colon cancer patients and is present but rare in other tumor types [87]. Investigators argued that benefit to colorectal cancer patients whose tumor exhibits MMR could cut across tissue origin classification, which motivated a 'basket-trial' based on selection of MMR phenotype [88]. These studies highlight how exploiting an intrinsic DNA damage repair deficits can be used to select patients for IO with the thought that these tumors are primed for neoantigen load. One study used TCGA lung tumor samples to comprehensively immunophenotype and analyze the links between DNA repair mutations, neo-antigen and total mutational burden, and tumor immune infiltration. High mutational burden tumors contained significantly increased infiltration by activated CD4 and CD8 T cells; furthermore, mutations in MMR genes, HR genes, or DNA polymerase *E* accurately predicted increased tumor mutational burden, neo-antigen load, and T cell infiltration [89].

Radiation can prime the immune system by both reducing tumor burden and increasing antigen presentation (i.e. immunogenic cell death) [90]. While cell killing provides a mechanistic rationale for combining RT with IO, we believe this perspective regarding the impact of radiation on the tumor is too narrow. An additive effect of PD-1 inhibition and radiation and preliminary studies suggest that a synergistic relationship exists[91, 92]. Preclinical data suggest radiation-induced signals in the TME may augment the immune system, as evidenced by increased antigen presentation, MHC expression, and antigen-specific T-cells [92]. The recruitment of active immune cells to an irradiated tumor has the potential to contribute to the efficacy of RT. Alternatively, a radiation primed immune response could augment the response to immunotherapy. This priming is supported by prolonged survival in mouse models undergoing combined checkpoint inhibition and radiation compared to mice undergoing monotherapy with either agent [56, 80, 81, 93, 94].

However, radiation-induced TGFβ activation, described above, likely compromises the extent of synergy in most of these studies. TGFβ activation by irradiated tumor cells can promote differentiation of immature CD14 myeloid progenitors to highly immunosuppressive myeloid derived suppressor cells (MDSC) by both increasing survival and promoting MDSC that make more TGFβ to suppress T cell proliferation and increase Treg [95]. MDSC are a heterogeneous population of poorly differentiated

myeloid cells that originate from CD14+ immature myeloid cells, that also give rise under physiological conditions to granulocytes, macrophages and dendritic cells (DC); their cell fate decisions are dictated by the tissue context to which they are recruited after release from the bone marrow [96]. MDSC levels are typically below 1% of peripheral blood mononucleocytes in normal healthy individuals but significantly increase during infection and pathological inflammatory conditions. As their name suggests, MDSC temper the degree of immune response. In cancer, high MDSC levels correlate with tumor burden, metastatic disease, and poor prognosis across carcinomas, and are increased in tumors, blood, lymphoid organs, and bone marrow in experimental cancer models [97-100].

Hence, targeting TGFβ is itself considered an immunotherapy and current thinking is that inhibiting TGFβ will likely promote responsiveness in combinations (**Figure 2**). Immunosuppression by TGFβ involves a phenotypic change in several immune cell types, including dendritic cells, tumor-associated macrophages, tumor-associated neutrophils, natural killer cells, MDSC, regulatory T cells , and cytotoxic T cells [101]. In the TGFβ–rich TME, dendritic cells shift into a tolerogenic phenotype, with reduced antigen presentation and ability to activate T cells. Although macrophages exist in a continuum of phenotypes, they are classically described as shifting from an inflammatory to a tumor-trophic phenotype to become tumor-associated macrophages that express pro-inflammatory cytokines at a reduced rate, while TGFβ and VEGF increase [102]. TGFβ can suppress the proliferation and cytotoxicity of NK cells, and reduce production of interferon gamma, which is an activator of macrophages and stimulates NK cells and neutrophils. Undifferentiated T cells can switch to a Treg phenotype in the presence of TGF-β, leading to the inactivation of effector and cytotoxic T cells.

Indeed, a bifunctional TGFβ trap and anti-PD-L1 fusion protein that simultaneously blocks both means of immunosuppression has shown efficacy in several mouse models, most recently in combination with radiotherapy [103, 104]. The dual acting agent synergized with radiation in an adaptive immune dependent fashion in poorly infiltrated murine model, in part by reprogramming the TME. Moreover, the agent has efficacy in blocking normal tissue toxicity that together speaks to the potential of broadening the therapeutic window, as has long been postulated [105].

TGFβ Regulation of the DNA Damage Response

Successful DDR requires the ability to recognize DNA damage, assemble the repair machinery, and execute repair; abrogation of any of these components decreases cell survival. Defective DDR is a hallmark of cancer, in which the mechanism or pathway deficit is often the basis for response to a specific type of therapy [61]. Therapeutic control is thus determined by the degree and type of DNA damage inflicted and the cancer cell intrinsic capacity to repair that damage, which were often empirically revealed by trial and error, e.g., use of cisplatin in head and neck squamous cell carcinomas or nitrosoureas in glioblastoma. But recognition of this key and lock interdependence for cancer therapy has led to the concept of personalized treatment based not on the cancer type but on identifying a specific defect in DNA damage repair that is an exploitable vulnerability to a selected cytotoxic therapy [106]. TGFβ's regulation of DDR in cancer provides a novel avenue to optimize care to cancer patients.

The unexpected requirement for TGFβ in the genotoxic stress program of normal epithelial cells was the impetus to actively pursue the translational potential of TGFβ inhibition in the context of radiation in preclinical cancer models. We initially focused on impaired ATM activity since ATM auto-phosphorylation and phosphorylation of histone H2AX (γ-H2AX) are important mediators of cellular recognition of DNA damage. Both are decreased in human and mouse brain, and in breast and lung cancer models treated with either TGFβ neutralizing antibodies or small molecule inhibitors of TGFβ signaling [66, 68, 107]. Decreased DNA damage recognition and ineffective cell cycle arrest are accompanied by greater radiation sensitivity (e.g., 10-70% less dose needed to reduce survival to 10% as measured by clonogenic cell survival) in most (35/43) breast, brain, head & neck, and lung cancer cell lines treated with a small

molecule inhibitor of the TGFβ receptor I kinase activity. Irradiation of tumors in mice treated with preclinical pan-neutralizing TGFβ antibodies also exhibit fewer γ-H2AX foci, indicating defective DNA damage recognition that leads to better tumor control [66, 68, 107].

ATM kinase is central to both non-homologous end-joining (NHEJ) and HR, which are the main repair pathways that cope with double-strand breaks. NHEJ repair pathway involving direct sealing of breaks ends is a fast process that is functional throughout the cell cycle except for mitosis. TGFβ also regulates the expression of *LIG4*, a critical mediator of NHEJ [108], either ATM or LIG4 deficits create radiosensitivity. HR uses homologous DNA sequences as repair templates is a slow and error-free process occurring in the S/G2 phases. ATM kinase needed for HR initiation and completion [109]. Many HR components are ATM substrates, including BRCA1, BLM, NBS1, MRE11, and CtIP. BRCA1 interacts with MRE11-RAD50-NBS1 (MRN) complex and in processing endonuclease CtIP to protect the DNA ends from the resection suppressor 53BP1 during HR. As TGFβ positively regulates BRCA1 by SMAD4 dependent transcriptional [110] and post-transcriptional mechanisms [37, 111]; hence, cells in which TGFβ is compromised have less efficient HR (**Figure 3**).

BRCA1 is post-transcriptionally suppressed by several microRNAs (miR) that control translation and mRNA degradation [112]. MiR-182 mediated downregulation of BRCA1 inhibits HR, leading to increased sensitivity to PARP inhibition [113]. TGFβ globally controls miRNA biogenesis through its regulation of Drosha and Dicer [114] and transcriptionally controls specific miRNAs. Genetic or transient inhibition of TGFβ increases miR-182, which in turn degrades *BRCA1* mRNA stability and inhibits translation, decreasing BRCA1 protein [111]. Antagonizing or overexpressing miR-182, but not other implicated miRs, abrogates TGFβ regulation of BRCA1. Therefore, HR s endorsed by TGFβ's control of BRCA1 [Moskwa, 2011 #18575] whereas inhibiting TGFβ decreases HR [Liu, 2018 #21177; Liu, 2021 #21698].

Moreover, we determined that the mechanism by which TGFβ inhibition impairs ATM kinase is indirectly due to miR-182 [37]. miR-182 also targets *FOXO3* (forkhead box protein O3), which is required for ATM auto-phosphorylation [116]. FOXO3 binds to ATM to facilitate kinase activity and promote DDR. Consistent with this, small molecule inhibitor of TGFβ receptor kinase no longer increases the radiosensitivity of cells in which TGFβ regulation of miR-182 is antagonized [37]. Thus, TGFβ has direct control of BRCA1 levels and indirect control of ATM activity via miR-182 [37, 111].

MicroRNAs can regulate hundreds of genes, which is evident in TGFβ regulated DDR genes. Isogenic SAS cell lines in which miR-182 was overexpressed or antagonized were treated as above with TGFβ and its inhibitor showed that TGFβ induction of BRCA1 mRNA depended on miR-182. In addition, TGFβ regulation of *MYD88*, *MRE11A*, *POLD4* and *PARP3* gene expression was a function of miR-182 expression. In contrast, TGFβ regulation of *CDKN1A* and *CCND2* were mir-182 independent, consistent with the presence of binding elements for TGFβ regulated SMAD in each. Thus, TGFβ has a broad impact on the DNA damage response by regulating expression of DDR components in both mir-182 dependent and independent mechanisms [115].

The clinical relevance of TGFβ contribution to the execution of DDR was validated in studies of human papilloma virus (HPV) positive head and neck squamous carcinoma (HNSC). Because HPV can only infect replicating epithelial cells, HPV targets TGFβ signaling components to allow squamous epithelial cells to proliferate. Viral protein E5 decreases phosphorylation of SMAD2 and nuclear translocation of SMAD4, as well as leading to progressive down-regulation of TGFβ type II receptor [117], E6 renders cells resistant to TGFβ mediated growth control by interacting and degrading both Smads and receptors that mediate signaling [118], and E7 interacts with SMAD2, 3 and 4 to significantly impede SMAD4-mediated transcriptional activity [119]. HPV-positivity is now ubiquitous in cervical cancer, and is a growing proportion of HNSC, particularly in young adults [120]. We can consider HPV-positive HNSC an

'experiment of nature' in which to investigate whether compromised TGFβ signaling in human cancer impairs DNA damage response and repair, as we had shown in preclinical cancer models. If so, one would expect sensitivity to genotoxic therapy.

Indeed, HPV-positive HNSC is remarkably response to standard of care cisplatin and radiotherapy [121]. To tackle this, we first confirmed lack of TGFβ responsiveness in HPV-positive HNSC primary tumors, patient derived xenografts, cell lines and transcriptomic data and showed that loss of TGFβ signaling compromises canonical HR and NHEJ [37]. We found that in addition to its impact on NHEJ, TGFβ controls execution of HR by indirectly regulating *BRCA1* levels via miR-182 [111]. miR-182 targets *BRCA1* message stability and translation in mouse and human cells [113], whereas TGFβ suppresses miR-182 [111]. Thus, when TGFβ is blocked in HNSC cells by HPV, or small molecule receptor kinase inhibitors, increased miR-182 suppresses BRCA1, and thereby compromises HR [37]. Wang and colleagues showed that that BRCA1 is also transcriptionally down regulated by CtPB1, which is regulated by SMAD4 transcriptional control from TGFβ [110] and that SMAD4 protein correlates with BRCA1 and RAD51 proteins in human HNSC [122]. Thus, TGFβ exerts profound control of HR repair by multiple mechanisms.

Cancer cells in which HR or NHEJ is defective shift to alternative end-joining (alt-EJ) repair, even though it is highly error-prone because it relies on microhomologies at processed ends leading to deletions and insertions. As a consequence of less robust DNA repair (i.e., fewer cells repair successfully) survival is reduced [123, 124]. Hence, cancer cells using alt-EJ are more sensitive to genotoxic chemotherapy or radiotherapy [125, 126]. HPV-positive HNSC cells showed hallmarks of alt-EJ; moreover, blocking TGFβ signaling in HPV-negative cells increases alt-EJ use. TGFβ-unresponsive HPV-positive HNSC cell lines are also more sensitive to the clinically available PARP inhibitor olaparib compared to TGFβ-responsive HPV-negative cancer cells, consistent with a non-mutation based synthetic lethality [37]. Thus, the biology of HPV-positive cancer provides compelling evidence that TGFβ signaling is key to how DNA damage is repaired, and that loss of competency creates deficits that can be exploited in combination with the current repertoire of genotoxic therapy.

While abrogation of TGFβ signaling by HPV affects cancer cell intrinsic responses, it is unclear how it affects the production and/or activation of TGFβ by these cancers. Indeed, TGFβ levels may actually be greater in HPV-positive cancers, which are insensitive to TGFβ, because they can still produce TGFβ that acts on stromal and immune cells when activated, as by radiation.

Translating TGFβ Biology for Cancer Therapy

Loss of TGFβ signaling competency for cancer cells has cancer cell intrinsic consequences. For example, TGFβ promotes cancer stem cell renewal in vitro and in vivo, which would be lost upon TGFβ inhibition [127, 128]. Moreover, TGFβ effects on cancer cell phenotypes facilitates invasion via appropriation of epithelial-mesenchymal transition, which is widely thought to contribute to metastasis [129]. As reviewed above, the mechanism by which TGFβ controls key transducers in the DNA damage cascade are complex [31, 34, 108] but poor molecular response to DNA damage increases cell death over survival; thus, inhibiting TGFβ during increases radiation response of in preclinical models of breast, brain and lung tumors [55, 56, 64-66, 107]. TGFβ inhibition disables execution of the DNA damage response and increases their radiosensitivity in most (38/43 tested in the author's lab) human and mouse cancer cell lines [37, 66, 107]. Blockade of TGFβ signaling also augments response to chemoradiation [64, 65].

The consequences of TGFβ control of DNA repair are perhaps best exemplified in HPV-positive HNSC remarkable response to standard of care cisplatin and radiotherapy [121]. Even though radiation would still lead to TGFβ activity that facilitates immunosuppressive and pro-tumorigenic phenotypes of resident non-malignant cells, the inability HPV-positive cancer cells to use TGFβ to repair genotoxic damage removes a barrier to therapeutic control. Although therapeutic resistance is often intrinsic to the cancer

cell, the less well appreciated is the role of the TGFβ in determining resistance to genotoxic therapy underscores a gap that hampers optimization of cancer treatment.

Together, the multiple mechanisms by which TGFβ acts in cancer cell intrinsic fashion provide a strong rationale for blocking TGFβ signaling will synergize with genotoxic therapies, of which radiation is the most widely used. DNA damage repair deficits represent an exploitable vulnerability in cancer [61], which are often empirically revealed by clinical trials, e.g., use of cisplatin in head and neck squamous cell carcinomas or nitrosoureas in glioblastoma. Recognition that the nature of specific deficit can be the basis for selection of treatment led to the concept of synthetic lethality [62]. The canonical example is synthetic lethality from PARP inhibitors in the context of HR deficits originally defined for germline *BRCA* mutations in breast and ovarian cancer patients [130]. The utility of PARP inhibitors has been expanded to cancers with somatic mutations in *BRCA1/2* or other HR components [63]. A similar synthetic lethality rationale can be used for cancers that are incompetent for TGFβ signaling.

There is wide variability in TGFβ activation among cancers and their competency to signal, for example, mutations in TGFβ pathway components are frequent in HNSC, colon cancer and pancreatic cancer [131]. To test whether the consequences of TGFβ on the DDR was evident in cancers besides HNSC, we interrogated the Cancer Genome Atlas (TCGA) with a signature of the genes induced following chronic TGFβ signaling and another signature consisting of genes involved in alt-EJ that include *POLQ*, which encodes polymerase theta, *PARP1*, and *LIG1*, all of which are decreased in HPV-positive HNSC compared to HPV-negative HNSC [37]. Notably the expression of the messenger RNA for these genes are actively suppressed by TGFβ treatment, and increased upon TGFβ inhibition, of cancer cell lines [115]. Consistent with this in vitro analysis, the TGFβ signature is significantly anti-correlated with alt-EJ signature in 16 of 17 carcinomas represented in the TCGA [115].

Cancers that have lost TGFβ signaling competency rely on alt-EJ, an inefficient and error-prone process, are predicted to be more responsive to genotoxic therapy, while those in which TGFβ is functional would be less sensitive. This prediction was tested by evaluating the relative survival of lung cancer, ovarian cancer, and glioblastoma patients in whom genotoxic therapy is standard of care (e.g., radiotherapy and platinum chemotherapy) as a function of the signatures. Remarkably, regardless of tissue of origin, patients whose tumors were alt-EJ high and TGFβ low had significantly longer overall survival or progression-free survival in response to genotoxic therapy [115]. These analyses, prompted by the greater sensitivity to chemoradiation exhibited in TGFβ incompetent, HPV-positive HNSC, provide compelling evidence that TGFβ plays a critical role in determining the response to radiation and other therapies that cause DNA damage.

Informed by the robust anti-correlation association with outcome, we converted the TGFβ and alt-EJ gene signatures into a custom NanoString panel. To validate that the NanoString assay accurately reports the biology we had defined in cancer cell lines, we used RNA from fresh specimens of HNSC primary tumor and patient-derived xenografts for expression profiling [132]. Unsupervised clustering of the gene expression signatures for TGFβ and alt-EJ clearly distinguished two groups of specimens characterized by the signature anti-correlation.

To functionally validate these signatures, explants of each tissue source were treated with TGFβ to measure pSMAD or irradiated and assayed 5 hr later for 53BP1 foci, a marker of residual or mis-repaired damage. TGFβ competency, as evidenced by induction of pSMAD2, was anti-correlated with unrepaired DNA damage (PCC=0.52; $p<0.001$), as we reported previously [37]. The TGFβ signature was significantly ($p<0.001$) correlated with pSMAD2, biological response to TGFβ, consistent with reporting a functional TGFβ pathway. The alt-EJ signature is significantly correlated with residual 53BP1 foci indicative of unrepaired DNA damage, consistent with reporting DNA repair competency. These data indicate that

function (e.g., pSMAD and residual DNA damage) and signatures are anti- correlated across a range of human HNSC specimens [132]. Thus, the TGFβ and alt-EJ gene signatures reflect the respective biological processes.

When TGFβ signaling is compromised, whether by pathway mutation, e.g., *SMAD4* or *TBR2*, or HPV-mediated epigenetic silencing [131], canonical repair pathways are compromised, and cells shift to alternative repair mechanisms. backup repair [123], which we refer to as alt-EJ, is also called microhomology mediated end joining [133-135], and theta-mediated end joining [136, 137]. The common feature among most studies using these different terms is the pattern of insertions and deletions with small regions of microhomology, high frequency of chromosome aberrations and translocations, and dependency on polymerase θ (POLθ), encoded by *POLQ* [126]. Indeed, our TCGA analysis showed that cancers in which high expression of the TGFβ signature is anti-correlated with low expression of the alt-EJ signature are characterized by the indel 6 mutation pattern, defined as >5-base pair deletions with overlapping microhomology at deletion boundaries with a mode of 2-base pairs [138]. This mutation pattern can be experimentally induced in cells in a POLθ -dependent manner [137]. Based on our data showing that *POLQ* knockdown further sensitizes cells in which TGFβ is blocked [37] and that TGFβ specifically regulates *POLQ* expression [115], our data is consistent with the definition of theta-mediated end-joining [139].

The consequence of TGFβ's dual cell autonomous roles endorsing canonical DNA repair and suppressing error-prone repair is to promote recovery from DNA damage from cancer therapy. When TGFβ signaling is lost or compromised, the decreased fidelity of DNA repair increases radiation sensitivity. We anticipate that blocking TGFβ and POLθ will synergize by compromising canonical repair and theta-mediated end-joining respectively.

Future Directions

The radiobiology of TGFβ provides novel insights into the ever-expanding universe of TGFβ biology. The root cause of this lies in radiation-induced rapid and persistent activation of TGFβ1. Radiation-induced activation provides a timed 'start' to investigate the TGFβ biology that rapidly ensues and dynamically evolves. The contribution of TGFβ to the response to radiation stems from three factors: first, ubiquity ranging from ligand distribution to receptors to signaling in all tissues; second, pleiotropy—TGFβ's regulation of many seemingly unrelated phenotypes; and three, centrality in cellular signaling networks that orchestrate self-renewal, cell fate and genomic integrity.

In the context of normal tissues, TGFβ mediates the phenotype of irradiated cells, initiates localized and systemic inflammation, elicits a regenerative extracellular matrix and directs cell fate. Together these processes can ultimately resolve damage and repair tissue. However, in the context of large-scale damage from high doses, pathology ensues from chronic TGFβ activity leading to pronounced inflammation, excessive ECM deposition, and tissue destruction culminating in fibrosis [140].

In cancer, the exuberant production and spurious activation of TGFβ promotes the malignant agenda by protecting cancer cells and corrupting control of angiogenesis, immune surveillance, and stromal support [11]. The subversion of TGFβ in cancer therefore represents a major challenge—can the detrimental effects of TGFβ activity in cancer be effectively targeted without compromising the TGFβ biology of normal tissues? The discovery that TGFβ endorses canonical DNA repair actively and suppresses error-prone repair [115] leads to several clinically important predictions:

- Robust TGFβ signaling is a mechanism of resistance to genotoxic therapy.
- TGFβ competency can be manipulated to capitalize on or create a specific DNA repair vulnerability.

- Inhibiting TGFβ signaling to increase chemo and radiosensitivity could be a new therapeutic strategy for patients identified based on this transcriptomic biomarker

Selectivity is likely the key. Implementation of TGFβ inhibitors in a manner that achieves isoform, spatial or temporal selectivity will likely succeed in the clinic. One clinical goal could be to neutralize TGFβ1 while preserving the activity of TGFβ 2 and 3, or to target activation in a cell type or context dependent manner, or to block TGFβ transiently during therapeutic interventions to increase DNA damage or release immunosuppression. Several recent reviews have compared both the diverse strategies and clinical outcomes of TGFβ inhibitors currently under investigation (see ref. [11, 141-143].

In the context of RT, which is widely and effectively deployed in multiple cancer types using a range of strategies, TGFβ opposes effective tumor control, suppresses immunity, and promotes dose-limiting toxicities. Identifying patients who will benefit by each mechanism will enable truly personalized therapy that targets the tumor vulnerability to overcome barriers to optimal patient outcomes and balance the risk/benefit ratio. The considerable pre-clinical and conceptual rationale for selective TGFβ blockade during radiotherapy is an opportunity that awaits realization.

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Figures

Figure 1: TGFβ opposes the mechanisms by which radiation controls cancer. The pleiotropic action of TGFβ in cancer is a major challenge to identifying the targets that will benefit patient outcomes. Radiation therapy induces TGFβ activation, which in turns promotes DNA repair, immune suppression and a permissive TME [5, 57, 69].

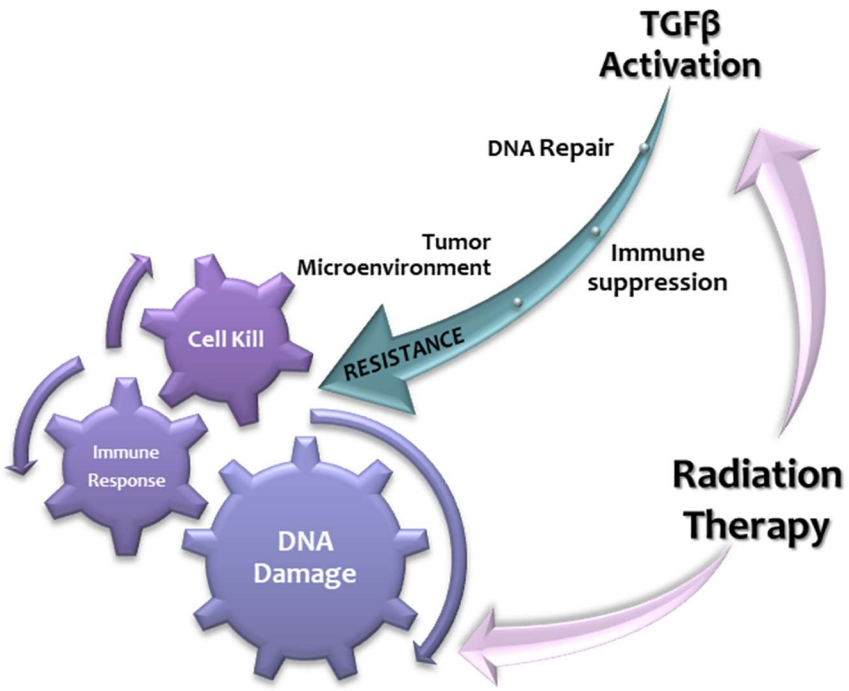


Figure 2: TGFβ regulation of myeloid differentiation in the tumor microenvironment promotes immunosuppression. TGFβ increases survival of monocytes and their differentiation into MDSC, at the expense of macrophages and dendritic cells. MDSC activate TGFβ to suppress cytotoxic T cells. Inhibition of TGFβ decreases immunosuppressive phenotype and alters the TME immune infiltrate. Modified from [95].

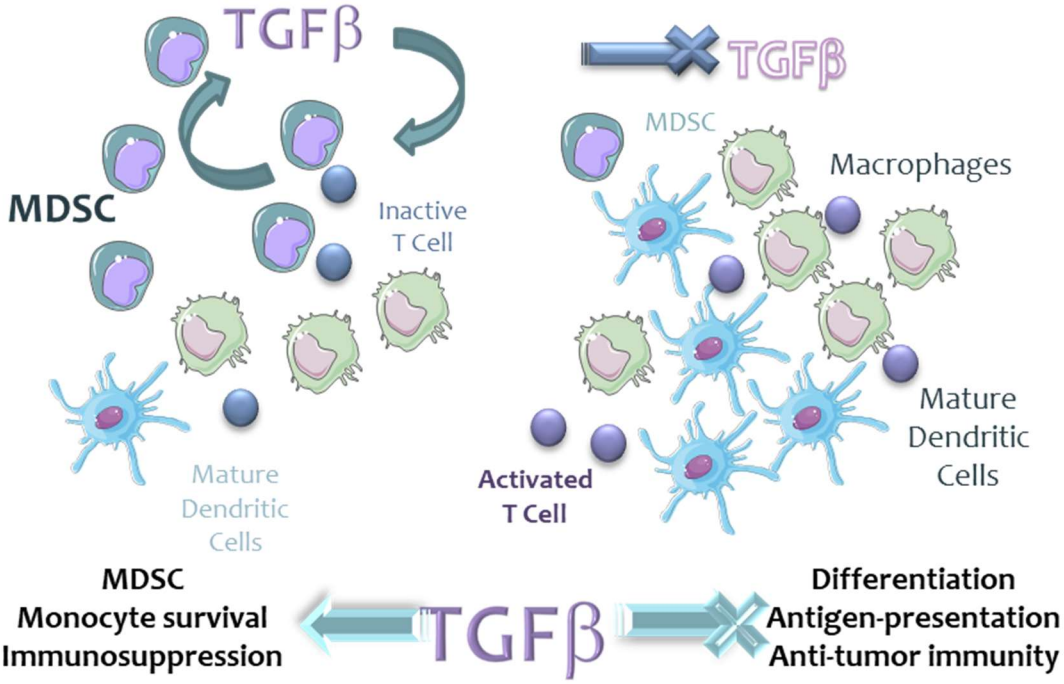
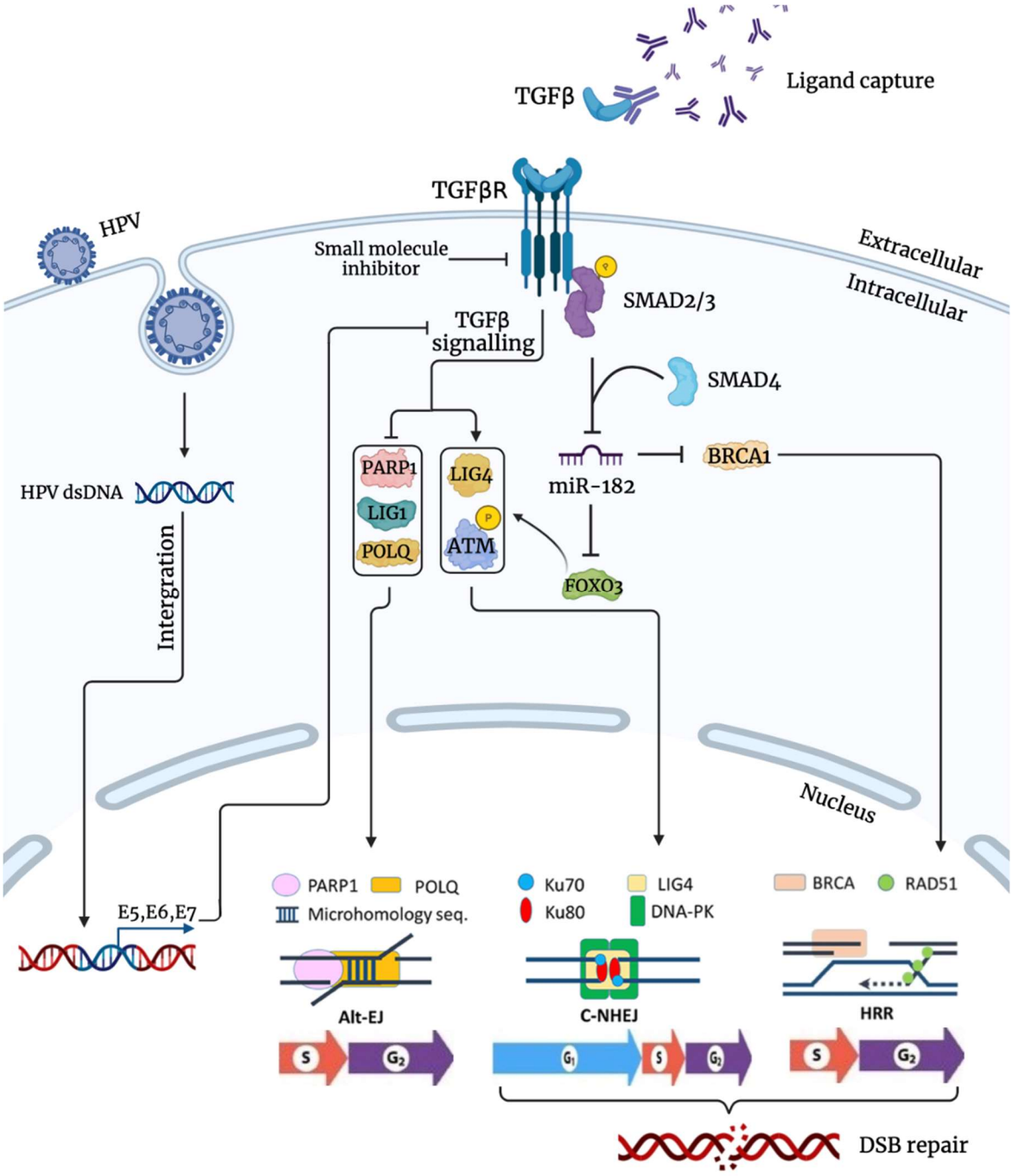


Figure 3: TGFβ regulation of the DNA damage response. Schematic connecting TGFβ to control of HR, NHEJ and alt-EJ via BRCA1, ATM and miR-182. Signaling from TGFβ requires extracellular activation in the tumor microenvironment. Loss of TGFβ signaling in cancers, whether by mutation, HPV infection, biological or pharmaceutical inhibition, impedes effective HR and NHEJ, and increases use of alt-EJ [37, 115]. SMAD dependent TGFβ regulated transcription is necessary for some events (e.g., miR-182, *LIG4* and *BRCA1*) but it is not known how loss of TGFβ increases expression of key alt-EJ genes (e.g., *POLQ*, *LIG1*, and *PARP1*). Thus, DNA repair competency is highly dependent on TGFβ biological activity and intrinsic cancer cell pathway integrity.



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