Rodriguez. *Plast Aesthet Res* 2023;10:67
**DOI:** 10.20517/2347-9264.2023.73

**Plastic and Aesthetic Research**

**Review**

**Open Access**

# Understanding how MSCs reverse radiation-induced fibrosis: HGF *vs.* TGF-B1

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**How to cite this article:** Rodriguez RL. Understanding how MSCs reverse radiation-induced fibrosis: HGF *vs.* TGF-B1. *Plast Aesthet Res* 2023;10:67. https://dx.doi.org/10.20517/2347-9264.2023.73

**Received:** 28 Jul 2023 **First Decision:** 26 Sep 2023 **Revised:** 6 Nov 2023 **Accepted:** 17 Nov 2023 **Published:** 4 Dec 2023

**Academic Editors:** Joseph M. Rosen, Roberto Cuomo **Copy Editor:** Yanbing Bai **Production Editor:** Yanbing Bai

## Abstract

Radiation induced fibrosis (RIF) can be understood as a form of chronic radiation-induced bystander effect (RIBE). It is a fibrotic process different than acute radiation syndrome (ARS), which is an inflammatory process that has different mediators and effector cells. It is triggered by Reactive Oxygen Species (ROS) activation of the matrix-embedded L-TGF-β complex. TGF-β acts by directing cellular processes that culminate in a fibrotic state. These include epithelial and endothelial mesenchymal transition (EMT and EnMT), G1 phase growth arrest, stimulation of fibrosis, and apoptosis, characterized by hypocellularity with a predominance of fibrocytes and myofibroblasts, fibrosis, and variable loss of tissue function. Fat grafting is the only clinically available tool to reverse RIF. The reversal of RIF is mediated by the mesenchymal stem cells (MSCs) embedded in the stromal vascular fraction (SVF) adipose tissue. The mechanism of action is the release of HGF (hepatocyte growth factor) by the MSCs into the surrounding RIF tissue. The HGF initiates a "mitotic growth program" that reprograms cell behavior. These changes include EMT and EnMT, stimulation of cell proliferation and morphogenesis, anti-apoptosis, downregulation of TGF-β, dissolution of fibrosis, and cell motility. The "mitotic growth program" culminates in tissue regeneration and reversal of RIF.

**Keywords:** HGF, TGF-β, RIF, radiation-induced fibrosis, fat grafting, MSC

## INTRODUCTION

Things that we have learned in medical school and residency about RIF (radiation induced fibrosis) include aphorisms such as "surgery is permanent, but radiation is permanent AND progressive". This presumes that radiation affects individual cells by causing damage to the cellular DNA, and that damaged radiated tissues do not heal well because of malfunctioning vasculature. Therefore, there is no cure for radiation damage except for the substitution of damaged tissue with healthy vascularized tissue (pedicled or free tissue transfer). The first report that avascular fat grafts restored RIF tissue to normalcy[1], and the demonstration by electron micrographs of regenerated tissues in fat-grafted areas[2], directly contradict our preconceived notions of radiation damage and how it can be reversed. In this article, Dr. Rigotti intimated that the healing effect of fat grafts on RIF is mediated by "stem cells". How these "stem cells" resident in fat graft heal radiated tissue is the subject of this review.

This article provides a simplified overview of how fat grafts regenerate RIF-damaged tissue based on published data. It is not an exhaustive review of molecular pathways but provides a general understanding of the principal cytokine dynamics of radiation damage, regeneration, and reversal of RIF. The cornerstone principles to understand are (A) RIF is an organized tissue response mediated principally by TGF-β (transforming growth factor beta) and (B) Fat Grafts reverse this damage in a process mediated by HGF (hepatocyte growth factor).

## RADIATION INJURY AND RIF

Radiation damage can be broadly divided into two categories- acute radiation syndrome (ARS) and the more chronic RIF. Although the precipitating agent is the same, they are different processes and not necessarily linked.

ARS is a type of inflammatory process that has its own set of cytokine and immune effector cells. When severe, it can progress to a "cytokine storm" that ends in systemic inflammatory response syndrome (SIRS). Although discussion of ARS is outside of the scope of this article, the reader is directed to other excellent reviews[3,4].

RIF, in contrast, is a fibrotic tissue response mediated by specific cytokines (mainly TGF-β) and immune effector cells (fibroblasts)[5]. Regardless, both ARS and RIF have in common the fact that they are mediated by cytokines and immune effector cells. In this article, we will focus on RIF.

This recent concept of damage mediated by cytokine messengers and effector cells has been referred to as Radiation-induced bystander effect (RIBE)[6] and is different than the previous consensus known as the "target cell theory". This theory held that the central event of Ionizing Radiation is damage to the cell DNA, and that the damage determined the cell's fate at the time of cell division.

### Target cell theory

According to this theory, ionizing radiation damages the DNA of individual cells. Each type of cell responds differently to a given dose of radiation depending on the effectiveness of its repair mechanisms against radiation damage. The repair may be effective, may end up in a mutation of the DNA, or it may be ineffective. The amount of damage is expressed as the cell goes into its mitotic cycle when the cell may (A) survive intact; (B) survive in a mutated state; or (C) may die [Figure 1]. Rapidly dividing cells, such as tumor cells, are more susceptible to radiation damage, but slowly replicating tissues can also express radiation damage later in time as the cells go into mitosis.

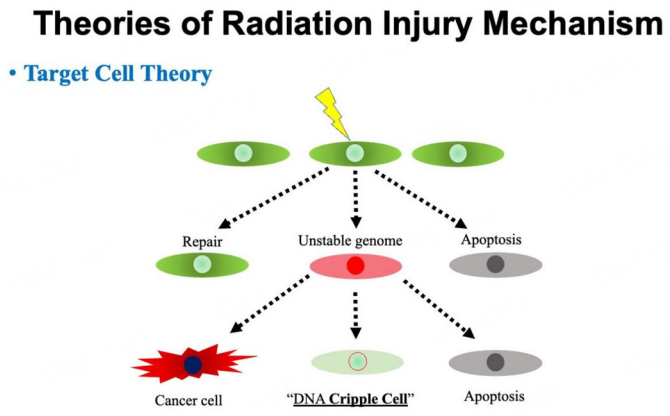


**Figure 1.** The target cell theory holds that when radiation hits an individual cell, it sustains damage. The cell attempts repair, or it may proceed to apoptosis. Sometimes it survives, but with damaged DNA and an unstable genome. The unstable genome may express as a cancer cell, a "crippled" no-functional cell, or may undergo apoptosis.

Under this rationale, the late radiation damage could be explained as the late sequelae of radiation injury on the "bystander tissue". Since those bystander cells had also been subject to radiation (at a sub-lethal dose for bystander tissue), the tissues could repair and recover from the sublethal dose, but the eventual scope of the damage would be revealed at the time of mitosis. This post-mitotic attrition in radiated fields would explain the progressive loss of normal tissue and function seen in interstitial tissues and blood vessels. This theory led to significant advances in Radiation Therapy, specifically, fractionation of dose delivery[7].

Historically, plastic surgeons focused on the blood supply of the radiated tissue as a means of helping the tissue heal. This rationale is intuitively appealing because of our focus on vascularized tissue flaps providing "fresh" blood supply to damaged radiated tissue. However, this is not a curative approach, but instead a palliative designed to substitute the damaged radiated tissue, not heal it.

As compelling as the target cell theory was, it did not explain several clinical observations. For example, how could one account for the severity of late damage seen in bystander tissues (the "bystander effect") that had been radiated at a sub-lethal dose, or for variations in late tissue damage (both in target cell populations as well as bystander tissues) not predicted by dose/response curves? Advances in single-particle microbeams to cell cultures provided an alternative explanation for these questions[8].

### Radiation injury bystander effects

In the early 1990s, with the advent of micro radiation techniques that could deliver radiation doses at the cellular level *in vitro*, investigators focused on the "bystander tissue" effects made startling discoveries that upended the target cell theory. For an excellent review on the subject, KM Prise's excellent review[9] is recommended.

Nagasawa *et al.* designed an experimental model where barrier strips were placed in a cultured cell plate so that only 1% of the cells were radiated[10]. An unexpected result was that 30% of the cells showed damage and nuclear DNA changes. Thus, other factors were at play rather than individual cell damage. Another series of experiments showed that the damage was not cell contact-dependent[11]. Cultured fibroblasts were irradiated and then placed in a well divided by a filter mesh that would allow culture media exchange but not cell passage. On the opposite side were cultured fibroblasts of normal phenotype that had not been irradiated. After a period of co-culture, the unirradiated side of the well also showed damage typical of irradiated cells,

including cytoplasmic and chromatid changes. For a schematic illustration of these experiments, see Figure 2.

This suggests that damaged cells were sending messages to adjacent cells that were, in turn, causing damage to these "bystander" non-irradiated cells. This is a phenomenon well known in immunology, where damaged cells release either damaged portions of the cell structure or specific biochemical signals that alert other cells to the damage. Some of these are called DAMPS or damage associated molecular pattern molecules[12]. Many DAMPs are nuclear or cytosolic proteins. When released outside the cell or exposed on the surface of the cell following tissue injury, they move from a reducing to an oxidizing milieu, which results in their denaturation. Others are direct products of energy transfer (ROS-reactive oxygen species). These compounds act as an alarm signal to the immune system. They create a "sense of danger"[3] in the tissues that results in an organized response by the tissue.

Because of these findings, the focus on countering the iatrogenic effects of radiation therapy has expanded from dosage delivery strategies to an effort to understand how the body responds to the radiation injury. The objective is to support and leverage the body's hemostatic response mechanisms.

Figure 3 illustrates the concept of cell damage in the "target cell theory" versus the concept of "RIBE". In this diagram, the effect of irradiation on the matrix is also added. As we will discuss later, the release of cytokines in the matrix, specifically TGF-β, plays a major role in RIF.

## TISSUE RESPONSE PATTERNS- INFLAMMATION, REGENERATION, REMODELING

The body's homeostatic response patterns to injury are well known to surgeons as "The wound healing curves". Under normal circumstances, the three phases of healing (inflammation, regeneration, and remodeling) each have their own set of triggering signals, messenger cytokines, as well as effectors and target cells, and temporal and spatial cues[13]. Each phase follows a common pattern, which is broadly divided into steps [Figure 4].

Step 1- For example, in the inflammatory pathway, the danger signals can be TNF-α (tumor necrosis factor), DAMPS (in the case of ARS), or PAMPS (pathogen associated molecular pattern molecules) in the case of infection. Step 2-These signals are picked up by the TLRs (toll-like receptors) embedded in the macrophage (M1 inflammatory type) cell wall. Step 3-The macrophage then acts as an amplifier of the signal by secreting inflammatory cytokines such as IL-6 (interleukin) that activate lymphocytes and activating other immune defense cells. Step 4- These immune effector cells (T lymphocytes, B lymphocytes) are the ones that attack pathogens or host bystander tissue. Step 5- Depending on local conditions, the effector cells send feedback to the sentinel cells to mitigate the amplification response (negative feedback loop) or accelerate it (positive feedback loop). They may also alter or reprogram host tissue cell function.

In penetrating or blunt trauma, a multitude of danger signals are released in a gradient over space and time. Sentinel cells react to these various signals in different ways. Consequently, a cascade of different tissue responses triggered over time results in an orderly progression from inflammation to regeneration and finally tissue remodeling.

Ideally, after the mitigation of the inflammatory reaction, the regenerative response takes over. Some of the signals for the regenerative response are the same "danger signals" and amplified cytokines from the inflammatory response. The regenerative response shares a proliferative function with the inflammatory reaction, but also carries with it an immune modulatory function that acts as a counterweight to the

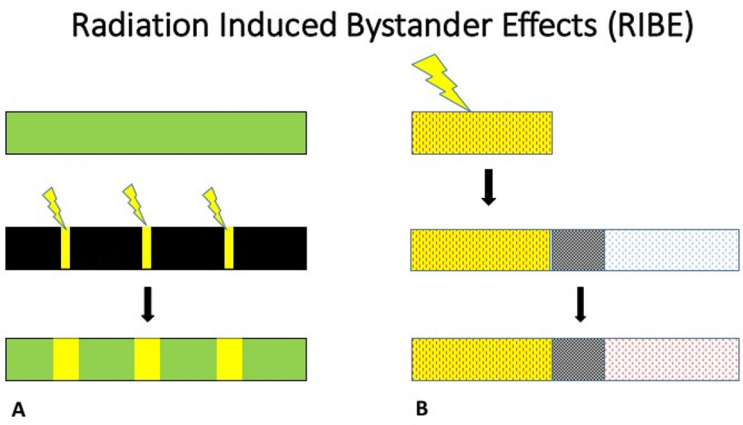


**Figure 2.** Experiments that illustrate RIBE. (A) A well of cultured fibroblasts (in green) was covered with Mylar dishes equipped with shutters (black) that left 1% of the cells exposed to irradiation (yellow strips). After several days of culture, 30% of cells showed changes typical of radiation damage[10]; (B) To determine if this effect was cell contact-dependent, a separate group of investigators irradiated cultured fibroblasts (red vertical hash pattern). After irradiation, they placed the irradiated fibroblasts in a well with unirradiated fibroblasts (blue divots, white background). The two cell preparations were separated by a permeable mesh (black mesh pattern) that allowed media exchange but no cell contact. After co-culture, the unirradiated fibroblasts showed cytoplasmic and chromatid changes typical of radiation damage[11].

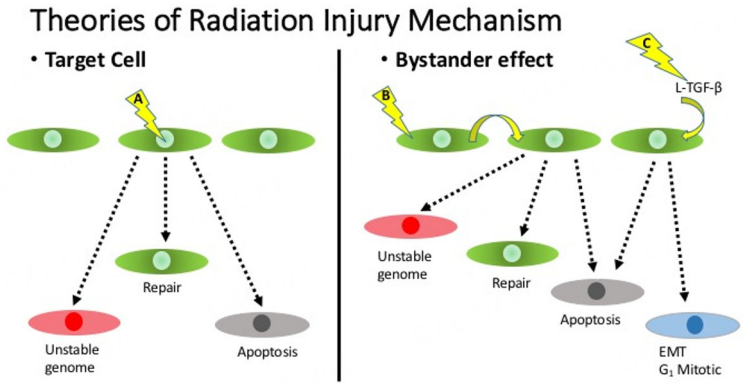


**Figure 3.** (A) "Target cell" mechanism of injury. Ionizing Radiation damages nuclear cell DNA. The cell responds by repairing itself successfully, whereas a faulty repair can result in survival with an unstable genome or apoptosis; (B) RIBE (radiation induced bystander effect)- Ionizing radiation damages any portion of the cell which can then release molecular compounds (DAMPS- damage associated macromolecular patterns) that act as "danger signals" to neighboring cells. These compounds may even cause chromatin changes in adjacent non-irradiated cells. The bystander cell responds by repairing itself successfully, whereas a faulty repair can result in survival with an unstable genome or apoptosis; (C) Ionizing radiation liberates matrix-embedded cytokines (TGF-β) that can then influence surrounding cells. Bystander cell behavior may then be "reprogrammed" by active TGF-β into undergoing apoptosis, EMT (epithelial to mesenchymal transformation), or mitotic arrest of the cell cycle at the G1 phase.

inflammatory reaction[14]. The main effector cell of the regenerative response is the Mesenchymal Stem cell[15-17].

The remodeling phase concludes the wound healing response. The effector cell of the remodeling phase is the fibroblast. Unlike the inflammatory or regenerative responses, it does not have much of a proliferative component. Like the inflammatory response, it has a prominent apoptotic function, but it is directed at paring off excess tissue created by the regenerative phase.

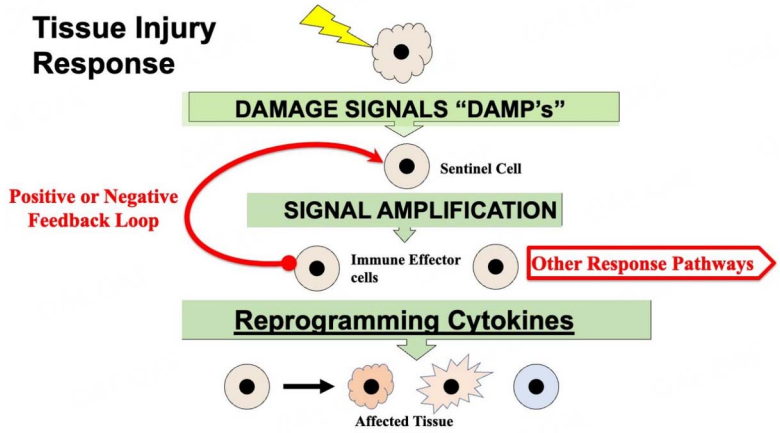


**Figure 4.** From the top: Step 1 When tissue is injured, damage signals (DAMPS, Extravasated blood, *etc.*) are released. Step 2 These damage signals are picked up by "sentinel cells", such as macrophages or mast cells. These cells initiate a cascade of events. Step 3 They can amplify the signal by secreting more alarm cytokines and chemoattractants. Step 4 The result is the recruitment of other immune effector cells. These can be granulocytes, Lymphocytes, dendritic cells, *etc.* These immune effector cells can (E) send negative or positive feedback signals. Alternately, they can trigger other response pathways and (F) reprogram Affected Tissue in different ways.

The main point of this brief discussion is to point out that the three main pathways share some common features with one but not the other pathway, and the three are linked temporally. But this complex relationship can be disrupted by an excess of one type of signal, or by the inability of a cell type to carry out its prescribed function. Another factor that can upset this interplay is whether the disorder is compartmentalized or generalized.

Finally, when one of these tissue response patterns goes out of control due to excessive signaling, or lack of effective feedback inhibition, the signal amplification process overrides other homeostatic checks and balances[18]. These events are known as "cytokine storms" or cascades. Many advanced disease states can be understood as falling within these abnormal tissue response patterns. Examples of an inflammatory "Cytokine storm" are sepsis and ARS. Although caused by different agents, they share the same final common response pathway- "Systemic Inflammatory Response Syndrome". An example of an unrestrained regenerative cascade would be a neoplasm, and an unrestrained remodeling phase can become a fibrotic state. Radiation-Induced fibrosis can be understood as one such cascade, precipitated by an overabundance of TGF-β.

## HOW THE TGF-β CASCADE CREATES THE TISSUE EFFECTS SEEN IN RIF

In RIF, the delayed tissue response is not as dependent on dosage and can be seen in patients where relatively low doses were administered. It is characterized by persistent fibrosis and varying degrees of loss of tissue function. The microscopic appearance typically consists of hypocellularity with a predominance of fibroblasts, an abundance of collagen, and blood vessel abnormalities.

Why is the delayed radiation injury pattern so specific, irrespective of irradiated tissue type? Why is the delayed tissue response different than other types of injury, such as penetrating trauma or blast injury?

### The role of TGF-β

TGF-β has been called "the master regulator of fibrosis"[19]. Several characteristics of this cytokine make it a good candidate for acting as a "master switch", specifically in the case of radiation injury.

TGF-β belongs to one of the major groups of cytokines (same family as BMP) and is important in embryonic axial development[20]. In the limbs, for example, it directs embryonic cells down terminal differentiation pathways along axial gradients as well as selective apoptosis; for example, it is responsible for the apoptosis and disappearance of the web membrane between the fingers.

In the adult, TGF-β is one of the dominant cytokines in the tissue remodeling phase.

Characteristics that make TGF-β a likely candidate for generating a "cytokine storm" are:

(A) TGF-β and its receptor are both manufactured by all cell types. Inside the cell, TGF-β is coupled to a ligand (LTBP- Latent TGF-β Binding Protein) and secreted to the extracellular space as an inert complex "L-TGF-β" (latent TGF-β).

(B) L-TGF-β is abundantly distributed throughout the extracellular space as a "matrikine" or cytokine that is embedded in the tissue matrix.

(C) The TGF-β/Receptor complex is "sticky", i.e., it stays bound for a period while intracellular messengers (SMAD 3,4) shuttle between the cell membrane and nucleus.

(D) Thus, every cell can be affected by and act as an amplification agent for TGF-β.

As a result of TGF-R stimulation, a suite of gene products are transcribed that activate a suite of intracellular pathways that culminate in altered cell behaviors[21]. These are:

- Differentiation of epithelial and mesangial cell lines to fibroblast and myofibroblast lineage.
- Directed cell senescence.
- Stimulation of apoptosis.
- Epithelial and endothelial mesenchymal transition (EMT and EnMT).
- Stimulation of collagen deposition.
- Stimulation of TIMPS (temporary inhibitors of metalloproteinases).
- Downregulation of metalloproteinases.

### How radiation damage differs from other types of trauma

In most types of trauma, such as blunt or penetrating trauma, a variety of "danger signals" (DAMPS, cytokines TNF-alpha, IFN, *etc.*) are released in a concentration gradient and a temporal sequence. In the case of RIF specifically, contact with ROS (reactive oxygen species) created by irradiation uncouples the L-TGF-β complex in the matrix of the entire irradiated field and releases the active TGF-β[22]. Because TGF-β is abundant and ubiquitous in the matrix, the TGF-β predominates in a uniform distribution over the entire irradiated area [Figure 5]. The activated TGF-β then binds to its cell membrane receptor (TGFR) in adjacent cells. It is as if the "normal" inflammatory danger signals and sentinel cell steps are paralleled by a pervasive,

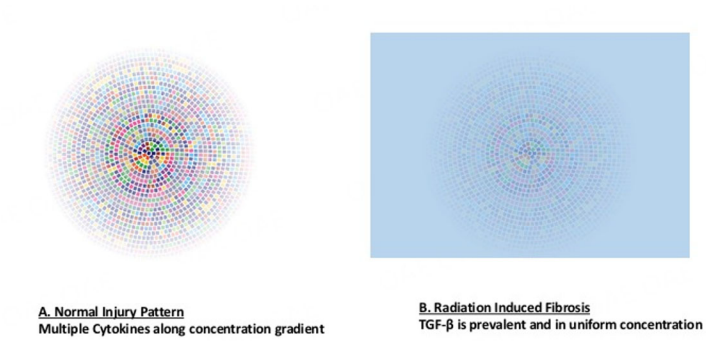


**Figure 5.** (A) After normal injury such as penetration, blunt trauma, *etc.*, a variety of cytokines (represented by the different colored dots) are released in a spatial concentration and temporal gradient, signaling cells in the immediate vicinity and attracting more distant cells. The multitude of signals elicits various responses from different cells orchestrating a balanced healing response that starts with inflammation, and progresses through regeneration and finally remodeling; (B) After Radiation injury, the L-TGF-β complex, which is widely distributed throughout the tissue matrix, is uncoupled and activated. Thus, the predominant cytokine signal is that of TGF-β (represented by the color blue). The cells and tissues in a radiated field are then driven to express TGF-β and culminate in specific cellular and tissue outcomes (Hypo cellularity, abundant fibroblast and myofibroblast populations, dense fibrosis, and blood vessel abnormalities).

uniform, and amplified signal: TGF-β. This triggers the TGF-β fibrotic response program. It has been described as an orchestrated tissue response[5].

The TGF-β fibrotic cascade in RIF can be described as follows:

Radiation Ionizing energy impacts the tissue and ROS species are generated. The L-TGF-β is a Redox sensor, so hydroxyl radicals created by radiation energy uncouple the L-TGF-β complex and active TGF-β is released. Active TGF-β is taken up by TGF-β receptors present in all cell types. The following instruction sets are then carried out:

### EMT, EnMT and blood vessel degradation

Under the influence of TGF-β, epithelial and endothelial cell types differentiate into mesenchymal cell types such as fibroblasts and myofibroblasts. In a normal healing situation, this might help during the remodeling phase, where initially disorganized and weak vascular networks are gradually consolidated into more robust and organized vascular trees and tissue matrix is laid down. In irradiated tissue, the persistent effect of TGF-β causes endothelial cells and epithelial duct cells to migrate out from their intraluminal position into the interstitium and they differentiate into fibroblasts. This cannibalizes the existing blood vessel endothelial cell population and damages the functional integrity of blood vessels.

### Terminal differentiation of cell lines

All affected cells in the resident tissue carry out a gene instruction set that blocks the DNA replicating system in the cell cycle $G_1$ phase. This locking of the mitotic cycle in the $G_1$ phase has two major consequences related to the ability of tissues to heal and replenish resident cell populations. First, various precursor cell types affected by TGF-β lose the ability to replicate and are directed to the fibrocyte/myofibroblast differentiation pathways and eventual senescence. Because of this, irradiated tissue has difficulty mounting a healing response to injury as its various precursor cell types cannot repopulate to create the necessary cell lines to carry out the various phases of healing. With the passage of time, cells senesce and die, and they are not replaced. This helps explain the observed hypocellularity of irradiated tissue and its relative inability to respond to injury.

### Blood vessel degradation

By upregulating MMP9 (type IV collagenase), TGF-β causes the degradation of collagens type 4 (basal lamina) and 7 (reticular lamina), which are prominent constituents of basement membranes. This effect is significant because it facilitates the migration of the endothelial cell to the extravascular space but also affects the structural integrity of the blood vessel. Together with the endothelial cell changes, these TGF-β effects on blood vessels help explain the increased permeability of irradiated vessels and structural abnormalities such as ectasia. Dr. Rigotti's electron micrographs [Figure 5] in his landmark article illustrate these phenomena clearly. MMP 9 itself also cleaves L-TGF-β, making more TGF-β available.

### Constitutive fibrosis

TGF-β also stimulates the production of collagen type III, the type found in ground substance. But in addition to the increased synthesis of collagen type III, there is also increased synthesis of TIMPs (temporary inhibitors of metallo proteinases). TIMPS inhibit collagen lysis. TGF-β also downregulates MMP1, which degrades native fibrillary collagen. The end result is a net overproduction of collagen (increased synthesis, decreased breakdown)[23].

TGF-β also stimulates the production of CTGF (connective tissue growth factor), a growth factor with a role in wound healing but which is seen in virtually all fibrotic states.

Once the specific cell behaviors triggered by TGF-β are well understood, a re-examination of Dr. Rigotti's electron micrographs[2] highlights the influence of TGF-β in RIF. In Figure 6, the authors observe lysosomes on an adipocyte and scattered intracellular debris. Both observations are linked to TGF-β directed apoptosis. The lysosomes are secreted by macrophages and contain lysosomal enzymes that digest apoptotic cells and create fragmented cellular debris in preparation for macrophage uptake.

The accumulation of collagen in the interstitial space is the result of TGF-β stimulation of Collagen type3, and inhibition of its breakdown by stimulation of TIMPs that inhibit collagenases active against collagen type3.

The duplication of the capillary vessel lamina and the separation between the endothelial cell and the pericyte are both the result of TGF-β stimulation of MMP2 and MMP9, both collagenases active against collagen type IV, the predominant collagen in basement membrane and basal lamina. This disruption in the basement membrane is usually the first step in the TGF-β directed EMT and EnMT, where disruption of the attachments to basement membrane prepares the endothelial cell to migrate out of the vessel and proceed to fibroblast differentiation. The Weiber-Palade bodies in the endothelial cell contain chiefly Von Willebrand factor and Interleukin-8 (IL_8). IL-8 is an inflammatory cytokine that can be induced by oxidative stress. In this setting, the weiber-palade bodies are related to the radiation oxidative stress but not necessarily to the TGF-β response.

## HOW DO FAT GRAFTS HEAL RADIATION INJURY?

The role of TGF-β has been understood by specialists in radiobiology for at least two decades. After the events of 9/11, the Federal government launched an initiative to counteract both ARI and RIF and dedicated $150 million to the effort. Lines of inquiry were directed at molecular antagonists to TGF-β, unfortunately without success[21].

Yet in 2001, Jackson *et al.* published a case report of successful treatment of RIF using fat grafting to the thigh of a woman who had had a limb-sparing rhabdomyosarcoma excision 20 years prior[1]. He reported a

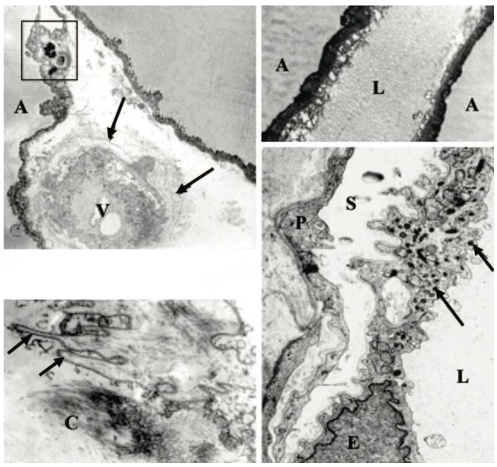


**Figure 6.** Figure text from the original article: "Ultrastructure of subcutaneous tissue after irradiation. (Above, left): A capillary vessel shows duplication of the basal lamina (arrows). An adipocyte shows lysosomes (inset) (original magnification, x2500) (A, adipocytes; V, vessels); (Above, right): A thick layer of collagen is visible between two adipocytes (original magnification, x5000). (Below, left): In the connective tissue, collagen accumulation and cell debris were visible, composed of membrane with fragments of external lamina (arrows) (original magnification, x7000); (Below, right): An endothelial cell (E) shows numerous Weibel-Palade bodies (arrows); A space (S) was often visible between the endothelial cell and a pericyte (P) (original magnification, x8000)."

long-term follow-up of three years with continued improvement of the tissues. Ironically, at a time when fat grafting was considered suspect by the ASPS, plastic surgeons had found an answer to a problem that radiotherapists considered to have no solution. That same year, Adam Katz and his colleagues at the University of Pittsburgh Medical Center[24] discovered that adipose tissue had a population of mesenchymal stem cells (MSCs) far in abundance than any other tissue, including bone marrow. In a seminal article published in 2007, Dr. Rigotti[2] attributed the healing power of fat grafts in irradiated tissue to the MSCs, which begged the question- How?

### MSCs and radiation injury

Since 2003, it has been shown that MSCs "home in" on radiated tissue and reverse both ARI[25] and chronic RIF, although as we have reviewed, they are different pathophysiologic processes. A great illustration of both methods of action is a series of experiments carried out by Lombaert *et al.*[26]. In the case of ARI, MSCs both repopulate the bone marrow with hematogenic precursors and modulate the SIRS inflammatory cytokine cascade. In the case of RIF, MSCs reverse the fibrotic TGF-β cascade and restore normal function to radiated cell populations. In the first part of the experiment, female rats (XX chromosome) were given a lethal dose of Total Body Irradiation (but with salivary glands shielded) and "rescued" by male rat (XY chromosome) MSC infusion. The male XY MSCs aborted SIRS and restored hematopoiesis in the bone marrow (XX stem and precursor populations). After a period of several weeks, the female rats had localized radiation to the salivary glands in doses that caused salivary gland RIF. They were then given doses of G-CSF (Granulocyte colony-stimulating factor) that release bone marrow MSCs into circulation. Interestingly, male (XY) labeled fibroblasts and myofibroblasts were detected in the interstitium of the salivary gland, but all functioning acinar and duct cells were female. Thus, the new functional and salivary duct and acinar cells were XX karyotypes that came from the radiated stock of cells. This is not a substitution of diseased tissue by a new population of cells but a reprogramming of RIF tissue into normalcy.

If TGF-β is the "master switch of fibrosis", is there a cytokine or group of cytokines that can reverse the effects of TGF-β? Our bias as Plastic Surgeons is to focus on blood supply, so investigations have focused on the ability of MSCs to regenerate blood supply. Consequently, VEGF (vascular endothelial growth factor)[27]

has attracted a lot of attention. But as we have seen, the effects of TGF-β are more global than just blood vessel damage and there is a more compelling candidate.

### TGF-β and HGF- the Yin/Yang of fibrosis[28]

HGF has been referred to as a direct antagonist to TGF-β induced fibrosis. HGF and TGF-β have been called the "Ying and Yang of Fibrosis" [Figure 7]. HGF directly inhibits the production of TGF-β and blocks the intracellular pathways from the TGF-β Receptor to nucleus at various points. In both kidney and liver injury models, levels of HGF relative to TGF-β coincide with the development of fibrosis versus its resolution. HGF has been proposed as a therapeutic possibility to treat fibrosis.

But if counteracting the effects of TGF-β is enough to arrest the progress of fibrosis, is it enough to regenerate tissue? We know that in the liver, many growth factors and extrinsic factors such as portal blood flow rates can influence rates of regeneration, but without HGF, there is no liver regeneration. Thus, at least in the liver, the primordial regenerating organ, HGF is indispensable[29]. Likewise, HGF receptor activation is indispensable for wound healing[30].

HGF[31]is a single inactive polypeptide secreted by mesenchymal cells and cleaved in the extracellular space by serine proteases into its active form. Like TGF-β, it is widely distributed in the extracellular matrix of most tissues. Its receptor is HGFR (hepatocyte growth factor receptor), which used to be known as the "C-Met" and was thought to be an oncogene. The receptor is expressed mainly by epithelial and endothelial cells. The receptor has a complex structure. The intracellular moiety has multiple branching points at which specific phosphorylation events trigger specific intracellular pathways. Like TGF-β, HGF is very important in embryogenesis. Throughout embryogenesis[32], HGF helps cells bud off from developing tissues and move towards and shape the complex architecture of prospective organs. It does promote EMT, like TGF-β. However, whereas TGF-β directs the cell to fibroblast differentiation, HGF allows the epithelial ductal cell or endothelial cell to proliferate and differentiate. HGF can direct cells to aggregate and form tubular structures with branching capabilities, which is important in creating both ducts and vessels. HGF also stimulates the production of MMPs active against collagen type3 (the most dominant in the interstitial tissue matrix). This helps migration and refashions the tissue matrix to accommodate the regenerating structures. Its function is similar in adult tissues after injury or during wound healing when lingering cells migrate into injury sites to restore and regenerate pre-existing structures. These activities - motility, cell survival, and proliferation embody a biological suite that is called the "invasive growth program"[33].

### HGF *vs.* TGF-β

The metaphor of HGF and TGF-β being the Yin/Yang of fibrosis in the context of fibrosis is perhaps incomplete as it does not account for the "invasive growth program" (regenerative response). Furthermore, the "invasive growth program" (regenerative phase) and the fibrotic (remodeling) phase are two of the major three response patterns of the body, with the inflammatory pathway being the third. The story of HGF versus RIF is the drama of healing unfolding in a dramatic, "reversed" way.

For example, in the case of RIF, there is such a uniform TGF-β amplified signal that the response unfolds deterministically from inflammation to its sclerotic end, like a biological winter. HGF can stimulate a regenerative response in RIF tissue that reprograms the entire tissue bed. Stem cells display their full armamentarium to create functioning structures of life where there were scars. The following are ways in which the "invasive growth program" counters "the master regulator of fibrosis."

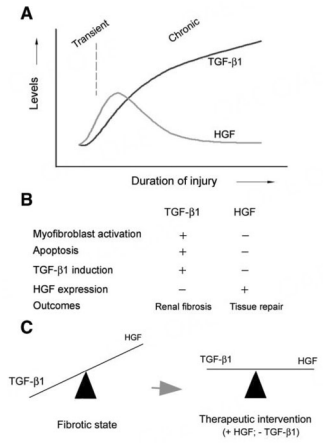


**Figure 7.** (From Liu[28]) Antagonism between HGF and TGF-β in the pathogenesis of renal fibrosis. (A) expression pattern of HGF and TGF-β in the diseased kidney. The duration of injury may determine whether the damaged tissues undergo recovery or fibrogenesis. Transient injury (dashed line) leads to a TGF-β/HGF ratio that favors HGF, resulting in tissue repair and regeneration, whereas chronic injury dramatically changes the TGF-β/ HGF ratio to favor TGF-β, leading to tissue fibrosis; (B) function of TGF-β and HGF in fibrotic kidneys. +: Promoting; -: suppressing; (C) therapeutic strategies for renal fibrosis. In the fibrotic kidney, the TGF-β/HGF ratio is out of balance, and TGF- 1 signaling dominates. Thus, therapeutic strategies should include a reduction of TGF-β activity and/or supplementation of HGF.

*Constitutive fibrosis*

TGF-β downregulates the production of collagenases and stimulates inhibitors of collagenases. The result is an accumulation of a densely fibrotic matrix. HGF, on the contrary, downregulates inhibitors of collagenases (TIMPS) and upregulates collagenases that break down matrix collagen. The net result is collagen lysis and the release of other trophic factors bound in the matrix. This allows refashioning of the matrix and facilitates cell migration.

*EMT and EnMT*

Both HGF and TGF-β stimulate endothelial and epithelial duct cells to migrate out of their vascular or ductal structures into the interstitial matrix. However, in the case of TGF-β, the cells are driven to terminal differentiation and change phenotype to mesenchymal fibroblast or myofibroblast, losing the ability to replicate. They progress to senescence. By contrast, in the case of HGF, during migration, cells are reprogrammed to the $G_0$ phase, thus able to replicate or differentiate into other cell lineages. The cell lineage is determined by other trophic factors, such as VEGF in the case of blood vessel formation. HGF also stimulates cells to form tubular structures that can bud and ramify. This is key to organogenesis, vessel formation, and duct formation. This is referred to as "morphogenesis", and without it, appropriate organ formation does not occur.

*Angiogenesis*

TGF-β degrades blood vessels by the twin mechanisms of EnMT and basement membrane degradation. It depopulates their cellular components (endothelial cells) as well as structural components (basement membranes). HGF also causes EnMT, but as mentioned before, cells are guided to form tubules, which are precursors of blood vessels. Although HGF and VEGF are both important to blood vessel formation and proliferation, HGF upregulates the production of VEGF and its receptor, and downregulates antiangiogenic factors. Furthermore, knockout of VEGF in the presence of HGF does not impair vascularization, as HGF stimulates vascularization via alternate mechanisms[34]. In contrast, knockout of HGF does reduce neovascularization significantly[35]. Thus, HGF is also paramount in blood vessel formation.

*Differentiation*

TGF-β locks cells into the $G_1$ phase of cell division cycle. This means the cell goes on to maturation and senescence, and the replication option is closed off. HGF, on the other hand, restores the cell's ability to go into the $G_0$ phase of the cell division cycle and repopulate depleted cell lines.

*Cell survival*

TGF-β triggers specific apoptotic pathways in cells. HGF, on the contrary, triggers specific anti-apoptotic pathways.

Putting it all together [Figure 8]

(1) When the tissue is irradiated, ROS break up the Ligand-TGF-β bond of Latent TGF-β and active TGF-β is released throughout the matrix in the irradiated area.

(2) TGF-β is bound to its receptor. It is a "sticky" bond, so the effect is prolonged, i.e., SMAD's 2and 3 shuttle several times between receptor and nucleus several times per single receptor activation.

(3) An intracellular messenger ($SMAD_{3-4}$ complex) is activated, goes into the nucleus, and results in transcription of a suite of gene products.

(4) These gene products affect cell behavior change as detailed below. These changes include (A) breakdown of blood vessel basement membranes; (B) this, in turn, facilitates migration of epithelial cells and endothelial cells, and terminal differentiation (EMT and EnMT), which results in depletion of epithelial and endothelial cells driving cells to the fibroblast/myofibroblast lineage (C) Apoptosis (D) deposition of collagen (E) upregulation of TGF-β itself. The result is the familiar clinical and microscopic picture of RIF: A hypocellular field dominated by fibroblasts/myofibroblasts and heavy collagen deposition with loss of normal tissue function.

(5) When the fat graft is injected, stem cells residing in SVF release HGF.

(6) HGF is bound to HGFR.

(7) (C-met oncogene). HGFR unleashes a variety of intracellular pathways and cellular interactions with macrophages and other immune cell types[36] that enable the "Mitotic growth Program". This HGF regenerative program is, feature by feature, the antithesis of the TGF-β fibrotic program.

(8) Feature by Feature comparison of HGF "mitotic growth program" *vs.* TGF-β Fibrosis instruction set. (A) HGF, like TGF-β, both cause EMT and EnMT. However, TGF-β drives cells to fibrocyte terminal differentiation, and the cell is blocked from DNA synthesis and duplication. On the other hand, HGF places cells in the S phase of the cell cycle, where DNA replication occurs, and the cell can undergo mitosis; (B) HGF, in conjunction with other cytokines such as VEGF or EGF, directs differentiation to different cell types, whereas TGF-β, by virtue of G1 cycle arrest, produces terminal differentiation; (C) Whereas TGF-β signals apoptosis, HGF signals anti-apoptosis; (D) HGF, to facilitate cell migration and refashion tissue matrix, stimulates collagen lysis. TGF-β results in fibrillary collagen deposition and a fibrotic matrix that can interfere with organ function; (E) HGF is a potent stimulator of angiogenesis (even independently of VEGF), whereasTGF-β impairs blood vessels by depletion of endothelial cells and disruption of basement membranes; (F) Finally, HGF itself causes downregulation of TGF-β. On the other hand, TGF-β upregulates itself.

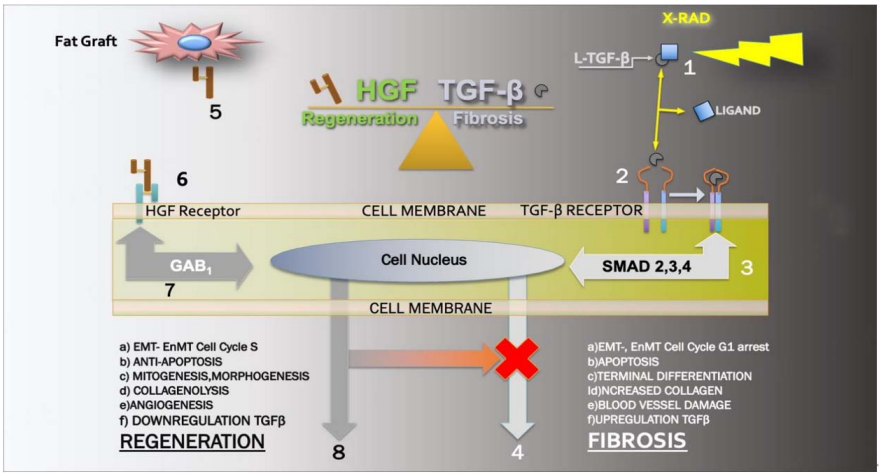


**Figure 8.** For a detailed description, see the text above. Briefly, 1-at the upper right hand of the image, one sees irradiation energy causing breakup of the TGF-β-Ligand complex with activation of TGF-β. 2- The TGF-β receptor is activated and 3- the SMAD complex initiates nuclear DNA transcription that results in 4 Secretion of various factors that affect various cellular behaviors (a, b, c, d, e, f), culminating in a fibrotic state. 5-When Fat Grafts are injected into the fibrotic tissue, SVF MSCs within the graft release HGF, 6- HGF receptor is activated and 7- the GAB1 intracellular moiety initiates a group of intracellular pathways that constitute the 8- "Mitotic Growth Program" (a, b, c, d, e, f) that in turn causes tissue regeneration and blocks TGF-β actions.

## HGF and MSCs

When one maps out the HGF "instruction sets", they look like the behavior of a Mesenchymal Stem cell, including a feature we have not discussed yet: the immune modulatory capabilities of HGF. HGF immunomodulatory pathways parallel in detail the mechanism of action of MSCs. For example, both HGF and MSCs generate immune modulatory programs by converting "classically activated" M1 Macrophages (pro-inflammatory) to M2 regenerative macrophage phenotype. Both HGF and MSCs work by stimulating the secretion of immunomodulatory amplifying signals IL-10 and PGE-2. They both suppress pro-inflammatory cell lines such as Th1, Dendritic cells and promote immune suppressive Treg and MDSCs (Myeloid Derived Suppressor Cells)[37].

Figure 9 illustrates the almost identical behavior of HGF and MSCs. The diagram on the left comes from an article by Nakamura *et al.*, one of the first authors to isolate and describe HGF[31]. One can see the intracellular moiety of the HGF Receptor triggering the "mitotic growth program" including (A) mitogenesis; (B) angiogenesis; (C) Morphogenesis; (D) anti-apoptosis, and (E) motogenesis. Although not depicted in the illustration, the same article also notes the immunomodulatory and antifibrotic properties of HGF. The diagram on the right comes from a nature review article on MSCs[38]. The parallels are strikingly close to identical to Nakamura's observations on HGF. MSCs are capable of (A) "Met"- (enhance proliferation); (B) angiogenesis; (C) differentiation; (D) anti-apoptosis; and (E) migration. The diagram also depicts the anti-inflammatory properties.

At the time of publication of the articles whose illustrations are compared above, there was little experimental corroboration of the theory that HGF is the "master regulator" for the MSC regenerative response in general, and RIF specifically. But now, several lines of evidence point strongly to this conclusion.

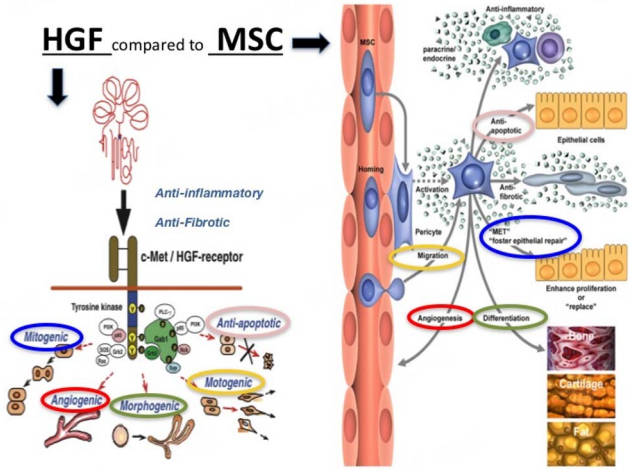


**Figure 9.** Similarities (represented by colored ovals) between the actions of HGF[31] on the left and MSCs[38] on the right. HGF, unlike TGF-β, is secreted only by mesenchymal cells like MSCs, but its receptor HGFR is expressed by many cell lines. The unique intracellular moiety of the receptor triggers many intracellular pathways that culminate in the "invasive growth program". These "instruction sets" code for behaviors that are strikingly like the effects of MSCs on target tissue. These include anti-apoptosis, angiogenesis, antifibrosis, migration ("motogenesis"), Mitogenic ("Met"), and anti-inflammatory effects.

MSCs may act mainly via HGF paracrine action. Although our preliminary understanding of stem cell action was based on the concept that the stem cells resident in the stromal vascular fraction of the fat graft, the more current of the role of the MSC is that it acts by homing in to injured tissues[39], paracrine action[40], and chemoattraction of helper cells to alter gene expression and behavior of cells in the injured zone. Although we cannot conclusively state that HGF is the "master regulator" of MSC reversal of RIF, experiments that promote or knockout HGF provide compelling evidence.

### Genetic modification (HGF gene promoter/knockout)

Several experiments have demonstrated that injection of HGF promoters directly into the liver or rat tail vein prior to irradiation conferred radiation protection[41,42]. However, the question of whether HGF was related to MSC action was answered more directly in several experiments. In a porcine model experiment, injections via coronary vein infusion were carried out one week after myocardial infarct. Animals were injected with saline only, null MSCs, and MSCs transfected with HGF or VEGF promoter genes[43]. Results showed improvement with null MSCs but more pronounced improvement with VEGF. Transfection with HGF showed even more significant improvements in survival, cardiac function, and reduced fibrosis compared to VEGF.

Specifically, in the case of radiation injury, a gene transfection study using a mouse model in which null MSCs and AD-HGF-modified (adenovirus gene transfection) MSCs were injected 6 hours after radiation to the lungs[44]. MSCs were effective in both the acute inflammatory and chronic fibrotic phases of radiation damage compared to the saline-only injection arm. However, the protective effect was much more marked in the AD-HGF-modified ASC group. The same author[45] repeated the same strategy for intestinal radiation injury. Results showed the same radioprotective effect of null MSCs, but enhanced radioprotective effect of AD-HGF Modified MSCs. There was also noted a systemic anti-inflammatory effect. The same radioprotective effect with HGF gene modification of MSC has also been noted in the liver[46].

Finally, it appears that in noncontact trans-well co-cultures, knockout of the HGF gene in MSCs impairs their ability to downregulate TGF-β expression in irradiated fibroblasts[47].

## SUMMARY

Plastic Surgeons have known for 2 decades that fat grafts reverse radiation damage. The effect has been attributed to MSCs in the fat graft's SVF. This article provides a broad overview of the molecular mechanisms involved in the healing of irradiated tissue by MSCs.

Radiation damage itself can be understood as having an acute inflammatory phase (ARS) and a chronic phase (RIF). Both phases are characterized by an organized tissue response that has its own set of alarm signals, sentinel cells, signal amplification, immune effector cells, and target cells. In the case of RIF, L-TGF-β (abundant in the matrix) acts as a redox sensor and releases TGF-β. TGF-β is known as the "master regulator of fibrosis". It precipitates a series of changes in cell behavior that culminate in hypocellularity with a predominance of fibrocytes and myofibroblasts in a fibrotic matrix. HGF counteracts the effects of TGF-β, and the interplay between the two cytokines has been referred to as the "Yin-Yang of Fibrosis". HGF is essential to all aspects of regeneration and triggers the "mitotic growth program" in addition to having immune modulatory properties. The activity profile of HGF is strikingly like that of MSCs. Investigators have used HGF gene promoters in MSCs that show HGF-enhanced MSCs demonstrate significantly enhanced radioprotective effects compared to unaltered MSCs. *In vitro* experiments show that knocking out the HGF gene in MSCs nullifies their radioprotection.

It is hoped that the insights gained from this review will propel new advances in regenerative fat grafting by focusing on the role of the fat graft as a delivery vehicle for HGF.

## DECLARATIONS

### Authors' contributions

The author contributed solely to the article.

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

The author declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.



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